Host response to polymicrobial infections in the lung

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Host Response to Polymicrobial Infections in The Lung

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Abstract

Lower respiratory tract infection associated with bacterial pathogens is a major cause of morbidity and mortality globally. As a result of the large volume of inhaled air, inevitably the lower respiratory tract is exposed to foreign substances, such as pathogens and allergens. Hence, to maintain homeostasis and sterility, the lower respiratory tract is equipped with multiple barriers and cells with distinct functions. Tightly connected ciliated epithelial cells are the first lining of the conducting airway, covered by a thin layer of mucus. Serving as physical barriers, mucus and the epithelium regulate mucociliary clearance and separate harmful particles from professional immune cells. On the other hand, the chemical barrier consists of secreted products, such as mucin, lytic enzymes, and antimicrobial peptides. In addition to epithelial cells, dendritic cells and macrophages are found in the lower respiratory tract. When epithelial cells are confronted with invading pathogens, bacterial ligands are recognised by receptors such as Toll-like receptors (TLR), which leads to sequential events, comprising activation of signalling cascades, expression and secretion of pro-inflammatory cytokines and chemokines, and recruitment and activation of professional immune cells. To bypass the immune response and facilitate successful infection, pathogens have evolved a wide range of virulence factors that subvert the host response at multiple steps.

The aims of this thesis were to investigate the differences in host responses to mono- and co-infection by common pulmonary pathogens *Staphylococcus aureus* and *Burkholderia cepacia* complex, to interpret the host receptor and signalling cascades targeted by *S. aureus*, and to characterise *S. aureus* immunomodulatory factors. Disruption of the intact airway epithelial barrier and cellular internalisation were observed with both species. *B. cenocepacia* J2315 activated TLR-mediated MAPK and NF-κB signalling pathways, subsequently eliciting a robust interleukin (IL)-8 production. However, when airway epithelial cells were co-treated with *B. cenocepacia* live bacteria and *S. aureus* supernatants, the pro-inflammatory response was subverted by Staphylococcal effectors. This anti-inflammatory effect was widely exhibited in *S. aureus* isolates tested, and was mediated via TLRs but not via the IL-1 receptor or the tumour necrosis factor receptor. TLR4 but not TLR5 was partially blocked by *S. aureus* secreted products, though the TLR4 agonist lipopolysaccharide did not affect IL-8 expression at a high concentration. The Staphylococcal effectors were preliminarily characterised as small, heat-stable, non-proteinaceous, and not cell wall-related factors.

This thesis demonstrates the complex nature of host response in a co-infection model and provides insight into a novel *S. aureus* immune evasion mechanism, as well as a therapeutic intervention of abnormal chronic inflammation.

List of abbreviations

AP1	Activator protein-1
BCAP	B-cell adaptor for PI3K
Bcc	*Burkholderia cepacia* complex
CCR	C-C chemokine receptor
CD	Cluster of Differentiation
CF	Cystic fibrosis
CFU	Colony-forming unit
COPD	Chronic obstructive pulmonary disease
CXCR	C-X-C chemokine receptor
CYLD	Cylindromatosis D
DC	Dendritic cell
DUBA	Deubiquitinating enzyme A
EGFR	Epidermal growth factor receptor
Erk	Extracellular signal–regulated kinase
Fab	Fragment antigen-binding
FAK	Focal adhesion kinases
FITC	Fluorescein Isothiocyanate
FLIPr	FPR2-inhibitory protein
FLIPrL	FPR2-inhibitory protein-like
FnBP	Fibronectin-binding proteins
Foxo1	Forkhead box protein O 1
FPR	Formyl peptide receptor
GSK	Glycogen synthase kinase
hBD	Human β-defensin
HBSS	Hanks' Balanced Salt Solution
Hlg	Haemolysin
HQNO	2-n-heptyl-4-hydroxyquinoline N-oxide
IFN	Interferon
IKK	Inhibitor of NF-κB kinase
IL	Interleukin
IM	Interstitial macrophage
IRAK	IL-1 receptor-associated kinase
IRF	IFN regulatory factor

IκBα	Nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor α
JAK/SOCS3	Janus kinase 1/Suppressor of cytokine signalling 3
JNK	c-Jun N-terminal kinase
LB	Luria-Bertani
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
LRTI	Lower respiratory tract infection
Luk	Leucocidin
Mal	MyD88 adaptor-like
MAPK	Mitogen-activated protein kinase
MCC	Mucociliary clearance
MOI	Multiplicity of infection
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-sensitive *Staphylococcus aureus*
MWCO	Molecular weight cut-off
MyD88	Myeloid differentiation primary response 88
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor κ-light-chain-enhancer of activated B cells
NLR	Nucleotide-binding oligomerization domain-like receptor
OD	Optical density
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PI3K/Akt	Phosphoinositide-3-kinase/protein kinase B
PRR	Pattern recognition receptor
PSGL-1	P-selectin glycoprotein ligand-1
PSM	phenol-soluble modulin
PVL	Panton-Valentine Leucocidin
Rab7	Ras-related protein-7
RhoA/ROCK/MLC	RhoA/Rho-associated protein kinase/ myosin light chain
RLR	Retinoid acid-inducible gene-I receptor
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SCCmec	Staphylococcal cassette chromosomal element
SCV	Small-colony variants
SIGIRR	Single immunoglobulin IL-1R-related molecule

SpA	Staphylococcal protein A
SPLI	Secretory leucocyte protease inhibitor
SSL	Staphylococcal superantigen-like proteins
T6SS	Type VI secretion system
TAB	TGF-β-activated kinase
TAK	TAK1-binding
TEER	Transepithelial Electric Resistance
TGF	Transforming growth factor
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TNFR	Tumour necrosis factor receptor
TRAF	TNF receptor associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain-containing adapter-inducing IFN-β

Chapter 1 Introduction

1.1 **Microbial infections of the lower respiratory tract**

1.1.1 Overview of lower respiratory tract infection

Lower respiratory tract infection (LRTI) is infection and inflammation caused by bacterial, fungal, parasitic or viral microorganisms in the trachea, bronchi and lungs. Depending on the rate at which it is spontaneously resolved by antibiotic treatments, it is defined as an acute or chronic illness, with cough and other symptoms including sputum production, dyspnoea, wheeze or chest discomfort/pain, with no alternative explanation (Woodhead et al., 2011). In contrast to relative short recovery period and less residual impact on the lower respiratory tract in the case of acute infections, insufficient but unrelenting immune responses and inefficient antibiotic therapies cause persistent chronic infections and exacerbations of pulmonary dysfunction. Chronic LRTI occurs often in patients with chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF) and non-CF bronchiectasis (Pragman et al., 2016). Children younger than 5 years and immunocompromised people are prone to LRTI and have severe illness. LRTI is the leading infectious cause and the fifth-leading cause of morbidity and mortality globally (Troeger et al., 2017). In 2015, mortality due to LRTI was 2.74 million in all ages and 0.7 million deaths in children younger than 5 years. Childhood undernutrition and air pollution are the major risk factors. According to the Global Burden of Disease Study, four aetiologies associated with LRTI are *Haemophilus influenzae* type B, *Streptococcus pneumoniae*, influenza and respiratory syncytial virus infection (Troeger et al., 2017). Although viral causes are the main causes for mild to moderate LRTI, bacterial pneumonia results in higher mortality compared to viral causes. *S. pneumoniae* leads to 55% of LRTI deaths in all ages as the most common cause and *Haemophilus influenzae* type B is mainly attributed to mortality in children younger than 5 years. Fortunately, vaccines against *Haemophilus influenzae* type B and S. *pneumoniae* are available and thus vaccination might reduce the occurrence of bacterial LRTI, in addition to antibiotics. Apart from the aetiologies mentioned above, the opportunistic pathogen *S. aureus* is another common causative agent in LRTI, particularly in hospital- and community-acquired settings, owing to the emergence of multi-drug resistance (Jones, 2010; Udo, 2013). Other common bacterial organisms are *Pseudomonas aeruginosa, Klebsiella pneumoniae, Escherichia coli,* and *Burkholderia cepacia* complex (Bcc) (Uzoamaka et al., 2017).

1.1.2 Dilemmas of antibiotic resistance

Antibiotics are the most commonly used treatment for LRTI. However, there are many limitations and adverse effects, including no effect on viral infections, rapid development of resistance, and side effects such as gastrointestinal disturbances. With the emergence of multi-antibiotic resistant isolates, effective treatments become more complicated, resulting in higher medical cost. The definition of antibiotic resistance is the ability of microbes to grow in the presence of antimicrobial agents at a concentration that would normally inhibit the growth (Brauner et al., 2016). Resistance is usually determined by measuring the minimum inhibitory concentration of planktonic (free floating) microorganisms, which acquire resistance either by chromosomal mutations or horizontal gene transfer of mobile genetic elements (Foster, 2017). Although antibiotic resistance evolves naturally, misuse or overuse of antibiotics accelerates this process. Therefore, it is critical to control and prevent infections, coupled with better stewardship of antibiotics.

S. aureus is a Gram-positive, opportunistic pathogen and readily acquires mobile genetic elements to develop antibiotic resistance using a variety of mechanisms (Haaber et al., 2017). Firstly, *S. aureus* acquires resistance to broad-spectrum β-lactams such as penicillin by acquiring plasmids that expresses β-lactamase. Soon after the clinical use of β-lactamase-stable derivatives such as methicillin, *S. aureus* adopted Staphylococcal cassette chromosomal element (*SCCmec*, encoding penicillin binding protein 2a) which confers resistance to β-lactams. These isolates are called methicillin-resistant *S. aureus* (MRSA) (Foster, 2017; Jevons, 1961). Alternative antibiotics choices include vancomycin (glycopeptide), tetracycline (tetracycline), fusidic acid (steroid-like antibiotic), and linezolid (oxazolidinone). However, resistant phenotypes towards these alternatives have emerged, posing significant challenges on *S. aureus* treatment (Gardete and Tomasz, 2014; Gu et al., 2012; McLaws et al., 2011; Schmitz, 2001).

Another opportunistic pathogen, Bcc is a group of at least 20 closely related Gram-negative, motile bacteria and a causative agent of LRTI, particularly in immunocompromised patients such as those with CF. *B. cenocepacia* and *B. multivorans* are the most common species isolated from CF patients, whereas *B. cepacia* is the most prevalent isolate in non-CF patients (Hauser et al., 2011; Kenna et al., 2017). Due to intrinsic or acquired antibiotic resistance, Bcc is very difficult to eradicate (Rhodes and Schweizer, 2016). Varied antibiotic susceptibility is seen between different species, within the same species, and between CF and non-CF isolates (Kenna et al., 2017). Factors such as efflux pumps, cell wall permeability and remodelling, expression of β-lactamases, and altered penicillin binding proteins all contribute to resistance to a large number of

antibiotics (Everaert and Coenye, 2016; Nitin Kumar et al., 2017; Malott et al., 2012; Podnecky et al., 2015). Antibiotics in clinical use against *Burkholderia* species infections include tobramycin (aminoglycoside), aztreonam and doripenem (β-lactams) (Scoffone et al., 2017; Waters et al., 2017). Combination treatments have also been proposed, such as moxifloxacin-ceftazidime-colistin, or ceftolozane-tazobactam (El-Halfawy et al., 2017; Mazer et al., 2017).

1.1.3 Polymicrobial biofilm communities in LRTI

In the natural environment or infection sites, microorganisms rarely exist in monospecies communities (Elias and Banin, 2012; Wolcott et al., 2013). There is a growing appreciation that bacteria, fungi and viruses co-exist in a polymicrobial biofilm community which is defined as a coherent community of multiple microorganisms embedded in a self-produced matrix adhering on a surface (Hall and Mah, 2017). Abundant evidence has shown that pathogens exist within biofilm colonies in the lower respiratory tract of patients with COPD, CF and non-CF bronchiectasis (Hassett et al., 2014; Marsh et al., 2014). Chronic infections caused by polymicrobial biofilms may eventually lead to the loss of pulmonary functions due to recurrent inflammation (LiPuma, 2010; Lynch and Bruce, 2013; Surette, 2014).

To switch from a free-floating mode of growth to a biofilm lifestyle requires environmental and genetic triggers as well as precise gene regulation. For example, subinhibitory concentrations of clindamycin contribute to upregulation of genes related to the stress response, attachment, and extracellular DNA production in *S. aureus*, and consequently lead to increased biofilm formation (Schilcher et al., 2016). Extracellular DNA is extended from the surface from active secretion or controlled cell lysis, and plays a critical role in attachment, maintaining integrity and protection (Okshevsky and Meyer, 2015). After initial attachment, cells in the colony produce an extracellular matrix containing exopolysaccharides, extracellular DNA, proteins and lipids (Mann and Wozniak, 2012). Subsequently, microbes multiply and mature into a more sophisticated community. Within the highly differentiated structure, spatial physiological heterogeneity occurs due to the steep gradient of oxygen, nutrients and waste. Compared to the cells in the outer layer, cells in the biofilm core are slow growing or even dormant (Wood et al., 2013). Finally, under shear stress or environmental factors (for example, antibiotics and immune response), microbes escape from the colony and disperse into the environment to find new optimal living space, which benefits microbial survival and dissemination (Kaplan, 2010).

Microbes dwelling inside biofilms are phenotypically distinct from their planktonic counterparts, and thus they differ in transcriptomes and proteomes (Freiberg et al., 2016; Resch et al., 2006). Among them, increased antibiotic resistance in biofilm phenotype may be the most important challenge (de Oliveira et al., 2016; Singla et al., 2012). Multiple factors contribute to modulating biofilm susceptibility to antibiotics, depending on the microbial strains and species, growing conditions, and developmental stage of the biofilm. The extracellular matrix confers the first layer of protection. A penetration barrier occurs where certain antibiotics such as vancomycin and chloramphenicol have limited penetration of Staphylococcal biofilms (Singh et al., 2016). The matrix also contains antibiotic-modifying enzymes such as β-lactamases, responsible for degrading β-lactam antibiotics (Bowler et al., 2012). Microorganisms are also protected against host response by molecules in extracellular matrix. For instance, alginate, the exopolysaccharide present in the matrix of *P. aeruginosa* biofilms, plays an important role in protecting biofilm cells from phagocytosis, even in the presence of interferon (IFN)-γ (Leid et al., 2005). Molecules responsible for adherence and aggregation in *S. epidermidis* biofilm matrix exhibit an immunomodulatory effect on murine macrophage, through inhibiting NF-κB activation and pro-inflammatory cytokine production (Schommer et al., 2011). Of note, dispersal of the biofilm structure by gentle ultrasonication without affecting cell viability restores phagocytosis and the pro-inflammatory response to *S. epidermidis,* even though dispersed bacteria are still coated with these molecules. This indicates that the secreted products in the matrix, as well as intact biofilm architecture, contribute to immune evasion. Overall, the matrix confers protection against antibiotic treatment and host defence to all the dwellers.

In addition to the protection by matrix, dormant cells in the core of biofilm remain at low growth and metabolic rate as a result of low oxygen, nutrient starvation and high levels of waste. Consequently, these cells are less susceptible to antibiotics that target fast-growing cells (Vandecandelaere et al., 2017; Walters et al., 2003). Additionally, the close proximity of cells to each other favours horizontal gene transfer via extracellular DNA or conjugation, which facilitates the exchange of multiple mobile genetic elements that may contain antibiotic resistance genes (Wintersdorff et al., 2016). In fact, biofilms confer a favourable environment as *S. aureus* biofilms exhibit up to ~16,000-fold higher frequency of conjugation compared to planktonic counterparts (Savage et al., 2013). The interactions are in a synergistic or antagonistic manner, and have impact on antibiotic resistance (Weigel et al., 2007). For example, in a dual-microbial biofilm model, *Moraxella catarrhalis* confers protection to *S. pneumoniae* by secreting β-lactamases, and in return, *S. pneumoniae* protects *M. catarrhalis* in a quorum sensing-independent pathway (Perez

et al., 2014). In contrast, antagonistic microbe-microbe interactions also promote resistance. *P. aeruginosa* secretes 2-n-heptyl-4-hydroxyquinoline N-oxide (HQNO), which suppresses the growth of *S. aureus* and ultimately reduces *S. aureus* viability (Orazi and O'Toole, 2017). HQNO leads to *S. aureus* shifting to small-colony variants (SCV) phenotype that is commonly associated with slow growing and persistence, whereby it promotes *S. aureus* biofilm resistance to vancomycin and other antibiotics that target biosynthesis (Proctor et al., 2006). Thus, polymicrobial interactions within biofilms play a critical role in regulating resistance and persistence.

Apart from the effect on antimicrobial resistance, an antagonistic interaction was observed in viability and biofilm formation in a *P. aeruginosa* and *S. aureus* co-culture model (Baldan et al., 2014). Long-term antistaphylococcal cephalexin treatment reduced *S. aureus* colonisation in infants and young children, whilst accelerated *P. aeruginosa* infection without improving clinical outcomes (Stutman et al., 2002). Notably, *S. aureus* is positively associated with 5-year survival in CF, while *P. aeruginosa* and Bcc associated with increased mortality and morbidity in children younger than 7 years old with CF (Liou et al., 2001; Nixon et al., 2001). In addition, *P. aeruginosa* and Bcc limit the microbiome diversity by suppressing the acquisition and persistence of other microorganisms, which correlates with worsen pulmonary function (Granchelli et al, 2018; Coburn et al., 2015).

Polymicrobial infections are also highly resistant to host immune systems (Bjarnsholt et al., 2009). However, due to the complex nature of microbe-microbe and host-microbe interactions, the features of host response to polymicrobial infections remain largely unclear. Different species seem to work in a cooperative manner inside the host. For example, an avirulent oral cavity commensal *Streptococcus* produces hydrogen peroxide (Ramsey and Whiteley, 2009). Once it is sensed by the oral pathogen *Aggregatibacter actinomycetemcomitans*, a genetic response is induced which contributes to the production of the complement resistance protein ApiA. As a result, this interaction facilitates the survival of both species from the killing by human serum. In addition, polymicrobial infections delay wound healing. For example, Dalton et al. (2011) transplanted mature biofilms composed of *P. aeruginosa, S. aureus, Enterococcus faecalis* and *Finegoldia magna* onto a murine chronically-wounded model, and observed significantly delayed wound healing at day 8 and increased antimicrobial resistance compared to wounds infected with *P. aeruginosa*.

Taken together, polymicrobial biofilm infections exhibit increased resistance to antibiotic and host defence, and delay wound healing, the net impact of which may lead to a worse outcome of patients. Therefore, understanding the mechanisms of host response to

polymicrobial infections may shed light on the persistent infections and inefficiency of antibiotics and immune clearance, as well as suggest novel clinical treatments.

1.1.4 Pathogenicity of Staphylococcus aureus

S. aureus is a commensal yet opportunistic human pathogen, harboured by about 30% of individuals (Wertheim et al., 2005). It is frequently isolated from the mucosal surfaces (such as nasopharynx, respiratory tract, urinary tract and gastrointestinal tract) and skin (van Belkum, 2016). While colonisation has different impacts to the host, it is a known risk factor of infections, as about 20% individuals harbor *S. aureus* persistently and a large proportion (60%) of individuals are colonised intermittently (Albrecht et al., 2015; Neha Kumar et al., 2015; Williams, 1963). Host symptoms may vary from asymptomatic carriage to severe infection, which is largely determined by the host response (Greenberg et al., 2018; Montgomery et al., 2014). Acquired in hospital and community settings, *S. aureus* is involved in life-threatening infections, including bacteraemia, sepsis, pneumonia, wound infections and indwelling device-associated infections (Tong et al., 2015).

In addition to the rapid evolvement of antibiotic resistance, *S. aureus* expresses a large arsenal of cell wall-anchored and secreted virulence factors to enhance the potential to cause infectious diseases (key factors are listed in Table 1.1). Two systematic reviews give detailed information on *S. aureus* virulence factors (Foster et al., 2014; Thammavongsa et al., 2015).

Adherence and internalisation are the prerequisite steps to establish infection. They rely on cell wall-anchored virulence factors such as clumping factor A and B (Elkhatib et al., 2015; Hair et al., 2010; Palmqvist et al., 2004), fibronectin-binding proteins A and B (FnBPA and FnBPB) (Foster, 2016), collagen adhesin (Zong et al., 2005), and SpA (Falugi et al., 2013; Jung et al., 2001; Pauli et al., 2014), through binding to extracellular matrix such as fibrinogen, fibronectin and elastin (Jonsson et al., 1991; Roche et al., 2004; Wann et al., 2000). Fibronectin serves as a bridge between $\alpha_V\beta1$-integrin on the host side and FnBPs on *S. aureus* surface, and thereby *S. aureus* enters into non-professional phagocytes by endocytosis (Sinha et al., 1999). Mutations in *fnb*A and *fnbB* significantly reduce renal *S. aureus* burden and mortality of mice, indicating that both FnBPs are crucial for Staphylococcal invasion (Shinji et al., 2011).

Table 1.1 Key immune evasion factors of *S. aureus.*

Name	Proposed Function	Target	Reference
Aureolysin	Immune evasion	C3	Laarman et al., 2011
Clumping factor A	Phagocytosis inhibition, adhesion	Fibrinogen, complement factor I, serum apolipoprotein E	Elkhatib et al., 2015; Hair et al., 2010; Palmqvist et al., 2004
Coagulase	Phagocytosis inhibition	Fibrinogen	Cheng et al., 2010
Extracellular complement-binding protein	Complement inhibition	C3d	Hammel et al., 2007a
Extracellular fibrinogen-binding protein	Complement inhibition	C3d	Ricklin et al., 2008
PSMs	Neutrophil chemotaxis, neutrophil lysis	FPR2	Geiger et al., 2012; Kretschmer et al., 2010; Surewaard et al., 2013
PVL	Neutrophil lysis	C5aR	Genestier et al., 2005; Spaan et al., 2013a
SpA	Complement and phagocytosis inhibition, inflammation, B cell superantigen, adaptive immune responses disruption	Immunoglobulin Fcγ and Fab, TNFR1, von Willebrand factor, EGFR	Dossett et al., 1969; Forsgren and Sjoquist, 1966; Gomez et al., 2004; 2007; Hartleib et al., 2000; Sasso et al., 1989
SSL10	Complement and phagocytosis inhibition	IgG, prothrombin, factor Xa, fibrinogen	Itoh et al., 2010; 2013; Patel et al., 2010
SSL3	TLR2 signalling inhibition	TLR2	Bardoel et al., 2012; Yokoyama et al., 2012
Staphylococcal binder of immunoglobulin	Complement inhibition	Immunoglobulin, C3 and factor H	Haupt et al., 2008
Staphylococcal complement inhibitor	Complement inhibition	C3 convertase (C3bBb)	Rooijakkers et al., 2005

The key virulence factor SpA (Figure 1.1) anchors on the cell wall and can be released into the extracellular milieu (O'Halloran et al., 2015). While SpA is responsible for immune evasion by binding to Fcγ and Fab domains of immunoglobulin (Deisenhofer, 1981; Graille et al., 2000), it contributes to pneumonia and inflammation in a murine model by engaging with TNFR1 and epidermal growth factor receptor (EGFR) (Gomez et al., 2004; Soong et al., 2011). On recognising TNFR1 and EGFR, SpA leads to activation of RhoA/Rho-associated protein kinase/ myosin light chain (RhoA/ROCK/MLC), followed by MAPK signalling and calpain activity in human airway epithelial cells (Soong et al., 2011). The cysteine protease calpains cleave junctional proteins occludin and E-cadherin (Chun and Prince, 2009), and thereby allow *S. aureus* and neutrophil penetration across airway epithelial cell monolayers through the paracellular junctions. However, in mice infected with *S. aureus* intranasally, calpain inhibitor but not EGFR inhibitor, increases Staphylococcal load, which indicates that SpA requires activation of EFGR and TNFR1 as well as proteolytic activity to invade airway epithelial barriers *in vivo*. Of interest, a later paper published shows that the *S. aureus* load is the same in *Tnfr1*$^{-/-}$ mice as in wild type mice (Ahn et al., 2014). An experiment that blocks both TNFR1 and EGFR should be done to confirm both receptors are critical for Staphylococcal invasion. Moreover, SpA seems to rely on TNFR1, EGFR and Fas ligands to induce IL-16 secretion in cluster of differentiation (CD)-4^{+} cells, which is mediated by Ca^{2+} flux and calpain activity (Ahn et al., 2014). Of note, neutralising IL-16 augments phagocytosis and alleviates tissue damage without affecting phagocyte influx, and thus IL-16 promotes pneumonia and excessive inflammation in SpA-mediated infections. Overall, due to a wide range of virulence factors, *S. aureus* is able to shift from innocuous colonisation to pathogenic infections; therefore, anti-adhesion therapies may be applied to prevent Staphylococcal infections (Arya et al., 2015).

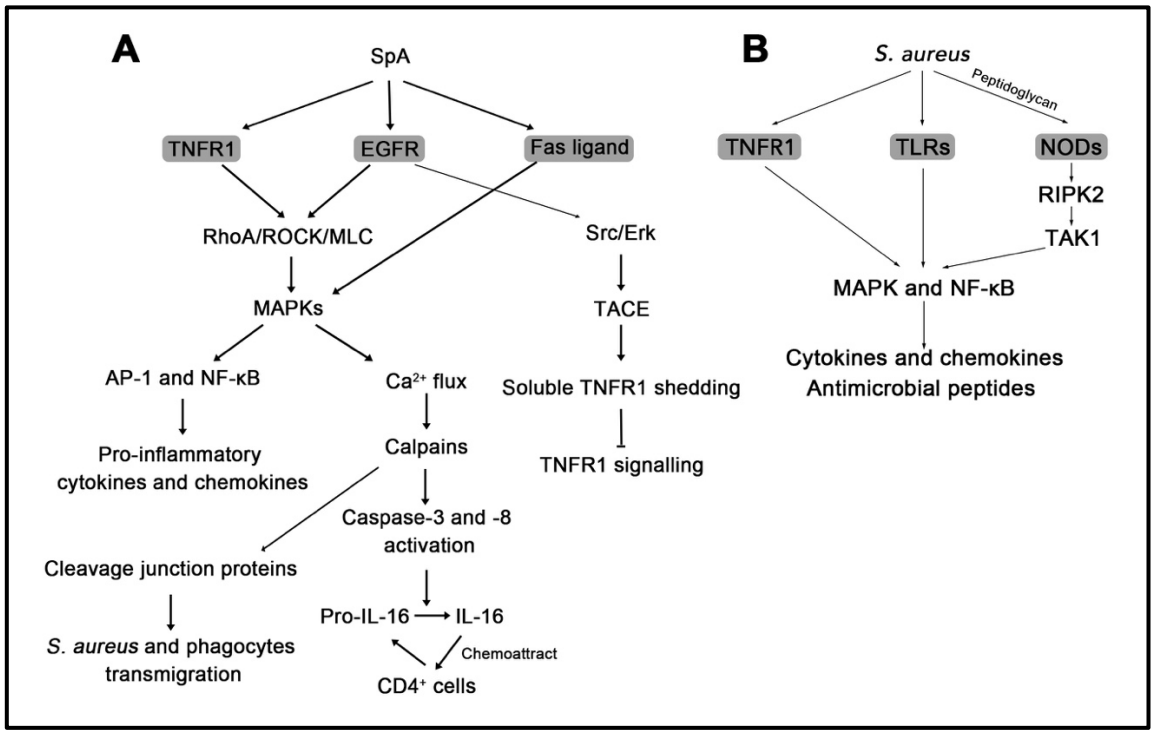

Figure 1.1 SpA targets and downstream host signalling.

(A) SpA can be recognised by multiple host receptors and modulate host signalling cascades in airway epithelial cells and $CD4^+$ cells, as described in Gomez at al., 2004; Soong et al., 2011; Ahn et al., 2014.

(B) Known signalling cascades in airway epithelial cells (Gomez and Prince, 2008).

Own original artwork.

Neutrophils are a major component in host defence against *S. aureus* and one of the first responders recruited to the site of infection. The migration of neutrophils, namely chemotaxis, relies on sensing the gradient of chemoattractant (Li Jeon et al., 2002) and adhesive interactions between endothelium receptors and neutrophil ligands (Choi et al., 2009). *S. aureus* possesses a wide range of ligands that bind to neutrophil surface receptors and disturb cellular homeostasis (Figure 1.2). For example, highly virulent community acquired-MRSA isolates express amphipathic phenol-soluble modulins (PSMs, Table 1.1) which recognise formyl peptide receptor (FPR)-2 on neutrophils (Kretschmer et al., 2010). PSMs elicit a robust pro-inflammatory response as well as neutrophil chemotaxis and subsequently lyse neutrophils. In contrast, *S. aureus* interferes with FPR in the opposite way. Staphylococcal FPR2-inhibitory protein (FLIPr) and its homologue FLIPr-like (FLIPrL) block FPRs and therefore subvert the neutrophil response to FPR agonists (Prat et al., 2006; 2009). Additionally, chemotaxis inhibitory protein of *S. aureus* blocks FPR1 and neutrophil complement receptor C5aR (de Haas et al., 2004). To hinder chemotaxis, Staphylococcal superantigen-like proteins 5 and 11 (SSL5 and

SSL11) recognise P-selectin glycoprotein ligand (PSGL-1) on neutrophils and consequently, disturb the interaction between PSGL-1 and P-selectin on endothelial cells (Bestebroer et al., 2007; Chung et al., 2007). Additionally, SSL3 (Table 1.1) binds to Toll-like receptor (TLR)-2 heterodimers and thereby inhibits TLR2-mediated pro-inflammatory response induced by Staphylococcal lipoteichoic acid and lipopeptides (Bardoel et al., 2012; Yokoyama et al., 2012). Staphopain and SSL10 (Table 1.1) target C-X-C chemokine receptor 2 and 4 (CXCR2 and CXCR4) respectively (Laarman et al., 2012; Walenkamp et al., 2009), which are critical for neutrophil activation and chemotaxis from the bone marrow (C. Martin et al., 2003). Furthermore, *S. aureus* promotes neutrophil lysis through secreting bicomponent pore-forming toxins which bind to the leucocyte membrane and form β-barrel pores that span the lipid bilayer (Alonzo and Torres, 2014; Spaan et al., 2017). Leucocidins consist of LukAB/HG, LukED, γ-haemolysin AB (HlgAB), γ-haemolysin CB (HlgCB) and Panton-Valentine Leucocidin (PVL, Table 1.1). LukAB/HG binds to CD11b subunit of the αMβ2 integrin on neutrophils (DuMont et al., 2013), and LukED binds to CXCR1 and CXCR2 on neutrophils and C-C chemokine receptor type 5 (CCR5) on T cells, macrophages and DCs (Alonzo et al., 2013; Reyes-Robles et al., 2013). HlgAB targets chemokine receptors including CXCR1, CXCR2 and CCR2, whereas HlgCB targets complement receptors C5aR and C5L2 (Spaan et al., 2014). Lastly, frequently exhibited in community acquired-MRSA isolates, PVL recognises C5aR and C5L2 (Spaan et al., 2013a). In addition to inhibiting neutrophil chemotaxis and complement activation, leucocidins (LukED and HlgAB) disrupt the erythrocyte membrane to sequester iron, and thereby acquire essential nutrients for proliferation inside the host (Spaan et al., 2015).

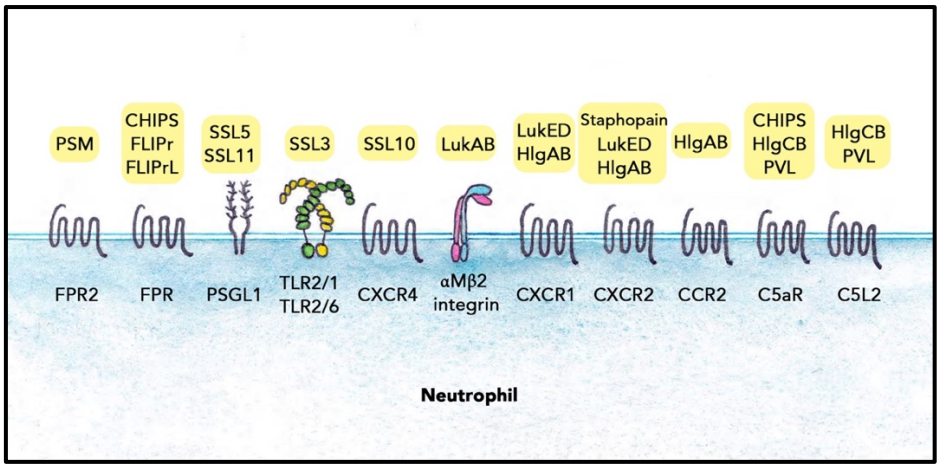

Figure 1.2 Staphylococcal inhibition of neutrophil activation and chemotaxis.

S. aureus subverts neutrophil extravasation and chemotaxis with multiple antagonists (yellow boxes) targeting neutrophil receptors (own original artwork).

Apart from SpA, *S. aureus* evades complement attack using other virulence factors. First, Gram-positive bacteria are protected by a much thicker peptidoglycan layer (20 nm) compared to Gram-negative bacteria (2.5 nm), which confers protection from membrane attack complex-mediated killing (Joiner et al., 1984). Capsular polysaccharides are reported to play an important role in evading opsonophagocytosis (Luong and Lee, 2002; Thakker et al., 1998; Nanra et al., 2013). Staphylococcal complement inhibitor recognises and blocks C3 convertases, which regulate all three complement activation pathways (Rooijakkers et al., 2005). Its homologues extracellular fibrinogen-binding protein and extracellular complement-binding protein (Table 1.1) bind to C3d and consequently inhibit C3 and C5 convertases (Hammel et al., 2007b; Jongerius et al., 2010a; Jongerius et al., 2007).

The fates of intracellular *S. aureus* and the infected host cells are determined by the host cell types, the isolates and the ratio of *S. aureus* to host cells (multiplicity of infection, MOI) (Kubica et al., 2008; Strobel et al., 2016). Following internalisation, *S. aureus* are rapidly engulfed into endosomes (in non-professional phagocytes such as epithelial cells, endothelial cells and fibroblasts) or phagosomes (in professional phagocytes such as neutrophils, macrophages and monocytes), which are intracellular compartments derived from the plasma membrane (Chi et al., 2014). Endosomes and phagosomes fuse with vacuoles and initiate the degradation of intracellular bacteria with proteases (Pillay et al., 2002), antimicrobial peptides (Rosenberger et al., 2004), reactive oxygen species (ROS) and reactive nitric species (RNS) (Fang, 2004), and are highly acidic (Hamaguchi et al., 2014). Nevertheless, *S. aureus* has the ability to survive in the hostile environment, escape into the cytoplasm and manipulate host cell programmed cell death.

To subvert lysozyme degradation of the cell wall peptidoglycan, *S. aureus* produces neutrophil serine protease inhibitors (Stapels et al., 2014). To reduce susceptibility to antimicrobial peptides, *S. aureus* exploits two distinct mechanisms. Due to the commonly cationic nature of antimicrobial peptides, the first tactic is to modify membrane lipids, which results in reduced negative membrane surface charge (Peschel et al., 2001; 1999). In addition, *S. aureus* secretes proteases including aureolysin and staphylokinase, which cleaves cathelicidin LL-37 and neutralises α-defensin, respectively (Jin et al., 2004; Sieprawska-Lupa et al., 2004).

S. aureus secrets antioxidants such as the carotenoid pigment staphyloxanthin, superoxide dismutase, Staphylococcal peroxidase inhibitor, catalase, and L-lactate dehydrogenase to reduce oxidative stress or nitrosative stress (Clauditz et al., 2006; de Jong et al., 2017; Gaupp et al., 2012; Mandell, 1975; Richardson et al., 2008). α toxin contributes to resistance towards oxidative burst through disengaging the close proximity

of internalised *S. aureus* to mitochondria in macrophages, and thereby protects themselves from mitochondrial ROS, caspase-1 and the antimicrobial metabolite fumarate (Cohen et al., 2018).

Despite phagosome pH being as low as 4 (Cachat et al., 2015), the pH lethal for *S. aureus* is 2 *in vitro* (Chan et al., 1998). In contrast to Gram-negative bacteria such as *E. coli,* phagocytosis and phagosome acidification play critical role in TLR-mediated responses by *S. aureus* (Ip et al., 2010). Unlike other MRSA and methicillin-sensitive *S. aureus* (MSSA) isolates, the highly virulent MRSA USA300 is able to survive in macrophage phagosome at pH 5 (Tranchemontagne et al., 2016). The increased virulence of USA300 is correlated with increased expression of regulatory elements including *agr, sarA*, and *saeRS* (Montgomery et al., 2008; Wang et al., 2007). The resistance of USA300 to phagocytosis appears to be triggered by acidification and other environmental cues in the phagosome, which leads to upregulation of *agr* gene locus expression (Tranchemontagne et al., 2016).

As part of the antimicrobial response, host cells initiate apoptosis once infected with bacteria and are subsequently engulfed by macrophages. Compared to other modes of cell death, apoptosis is preferable and non-inflammatory through multiple mechanisms (Szondy et al., 2017). However, *S. aureus* either evades apoptosis through EsxA, a substrate of ESAT-6-like secretion system or switches to necrosis in an *agr*-dependent manner (Korea et al., 2014; van Krüchten et al., 2018). In contrast to apoptosis, necrosis involves the disruption of the plasma membrane and release of cellular contents. Thus, it is highly pro-inflammatory and detrimental to the host, leading to tissue damage (Edinger and Thompson, 2004).

Taken together, the opportunistic pathogen *S. aureus* is capable of shifting from commensal to pathogenic and fully equipped with a plethora of virulence factors to survive and persist in the host.

1.1.5 Pathogenesis of Bcc

As mentioned above, Bcc species are opportunistic pathogens that mainly affect immunocompromised groups such as CF patients, and one of the major challenges is its intrinsic antibiotic resistance. The clinical manifestations of *Burkholderia* colonisation can vary greatly and is unpredictable. Whilst some patients exhibit asymptomatic chronic infection, about 20% of patients have a life-threatening exacerbation of respiratory function among CF and non-CF patients (Isles et al., 1984; Hauser and Orsini, 2015). The

‘cepacia syndrome’ is characterised by pyrexia, rapidly progressive pulmonary function decline, sepsis and bacteraemia.

Although only a small proportion of CF individuals (2.7%) are colonised by Bcc, the acquisition is a major concern among CF patients, their caregivers and families. It has been acknowledged since the 1980s that certain epidemic strains (for example the ET12 clone and PHDC strain of *B. cenocepacia*) are highly transmissible among patients and even from patients to non-CF individuals (LiPuma et al., 1988; Govan et al., 1993; Holmes et al., 1999). Thus, a strict cohort segregation for CF patients harbouring Bcc was implemented worldwide; however, it caused fear and loneliness to worsen the psychological conditions of these individuals (Duff, 2002). Due to the strict infection control measures, the new acquisition of *B. cenocepacia* dropped dramatically after 1995 and *B. cenocepacia* became less predominant among CF individuals (LiPuma, 2010; Zlosnik et al., 2015). Survival analysis reveals that patients colonised with *B. cenocepacia* have a significantly worse outcome compared to *B. multivorans* (Zlosnik et al., 2015).

Nevertheless, new acquisition of Bcc still occurs in spite of segregation practice. In Queensland, Australia, 75% new cases of the total incidence of Bcc colonisation occurred during in the period 2001-2011. The isolates reveal high genetic diversity and the majority of patients (85%) are associated with unique strains (Ramsay et al., 2013). This indicates the sporadic acquisition of Bcc from independent sources in the post-segregation period, most likely from the environment and can be affected by environmental factors such as the amount of rainfall (Peddayelachagiri et al., 2016; Ramsay et al., 2013).

Bcc species are known to breach the epithelial barrier, leading to bacteraemia, as well as trigger an exacerbated proinflammatory response (Downey et al., 2007; McClean and Callaghan, 2009; Saldias and Valvano, 2009). Moreover, Bcc is capable of survival within bronchial epithelial cells and professional phagocytes or mucus extracellularly in the lumen (Schwab et al., 2014). Intracellular *B. cenocepacia* J2315 (ET12 clone) is sequestered in *B. cenocepacia*-containing vacuoles that are fused with early endosomes. In contrast to heat-inactivated bacteria, which are rapidly degraded in the phagolysosome, live bacteria subvert the phagocytic pathway by delaying phagosomal maturation and fusion with the lysosome by up to 6 h, and thereby maintaining the vacuole at pH 6.4 (Lamothe et al., 2007).

Furthermore, the assembly of Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex on live *B. cenocepacia*-containing vacuoles is delayed compared to heat-inactivated *B. cenocepacia* (Keith et al., 2009). NADPH oxidase contributes to ROS by producing the precursor superoxide. This results from the manipulation of Rho family

GTPases by type VI secretion system (T6SS)-derived effectors (Table 1.2) (Rosales-Reyes et al., 2012b). Morphology alteration is observed in murine macrophages by live *B. cenocepacia*, which involves deactivation of Rho GTPases in a T6SS-dependent manner (Flannagan et al., 2012; Rosales-Reyes et al., 2012b). The T6SS-derived effector TecA deamidates Rho GTPases and leads to cytoskeleton disruption and inflammation (Aubert et al., 2016).

While it has been shown that Bcc species readily form strong biofilms *in vitro* (Caraher et al., 2007; Scoffone et al., 2016), Schwab et al. (2014) have reported that only single bacteria or small clusters are observed either in phagocytes or mucus in bronchial sections from CF lung tissues. This may indicate that biofilm formation is not a virulence factor associated with *B. cenocepacia* infections.

To facilitate motility and invasion, Bcc is equipped with whip-like polar flagella (Table 1.2) (Tomich et al., 2002). The flagellin filament of flagella is specifically recognised by TLR5 and the major ligand that induces a pro-inflammatory response in airway epithelial cells (de C Ventura et al., 2008; Hayashi et al., 2001). Consequently, blocking the association between flagellin and TLR5 improves lung injury in CF (Blohmke et al., 2008).

Another major virulence factor, lipopolysaccharide (LPS, Table 1.2) can activate TLR4, leading to a cytokine/chemokine response through activation of MAPK and NF-κB (Bamford et al., 2007). Compared to other Gram-negative bacteria, Bcc LPS has unique properties and confers protection against antibiotics and antimicrobial peptides (Loutet et al., 2006; 2010; Vinion-Dubiel and Goldberg, 2003). The positively charged 4-amino-4-deoxy-arabinose is present in the lipid A and core oligosaccharide, which neutralises the anionic charge of phosphate residues in LPS (De Soyza et al., 2008). Consequently, 4-amino-4-deoxy-arabinose contributes at least partially to the resistance to cationic antibiotics and antimicrobial peptides (Hamad et al., 2012; Scott et al., 1999).

In addition, clinical Bcc isolates are commonly coated extracellular by mucoid exopolysaccharide (Table 1.2) (Herasimenka et al., 2007). Exopolysaccharide are associated with chronicity, and confer protection by inhibiting neutrophil chemotaxis and production of ROS, as well as scavenging ROS (Bylund et al., 2006). However, CF individuals exclusively infected with nonmucoid isolates (such as *B. cenocepacia* J2315) is correlated with a faster rate of lung function decline compared to patients infected with mucoid Bcc (Zlosnik et al., 2011).

Taken together, Bcc species are highly virulent and readily to adapt from natural environment to dwell inside the human host with the assistance of a wide range of virulence factors.

Table 1.2 Key virulence factors of Bcc

Virulence factors	Functions	Reference
Cable pili and the 22-kDa adhesin	Targeting cytokeratin 13 and transmigration across the airway epithelium, inflammation	Urban et al., 2005; Goldberg et al., 2011
Exopolysaccharides	Phagocytosis inhibition, chemotaxis inhibition, ROS inhibition	Bylund et al., 2006; Conway et al., 2004
Flagella	Motility, adhesion and invasion, and inflammation	Tomich et al., 2002; Urban et al., 2004
LPS	Inflammation, intrinsic antibiotic/antimicrobial peptides resistance and phagocytosis inhibition	Silipo et al., 2007; Loutet et al., 2006; Vinion-Dubiel and Goldberg, 2003; Saldías et al., 2009
Peptidoglycan-associated lipoprotein	Adhesion and inflammation	Dennehy et al., 2017
Quorum sensing	Regulation of the expression of siderophore, zinc metalloproteases, swarming motility and biofilm formation	Lewenza et al., 1999; Huber et al., 2001; Kooi et al., 200
RpoE	Phagocytosis inhibition, stress response regulator, and polymyxin B resistance	Flannagan and Valvano, 2008
RpoN	Phagocytosis inhibition, biofilm formation, motility, nitrogen metabolism and exopolysaccharide synthesis	Saldias et al., 2008; Fazli et al., 2017; Lardi et al., 2015; Liu et al., 2017
Siderophores and $Ftr_{Bcc}ABCD$	Iron uptake	Mathew et al., 2014; Thomas, 2007
T6SS	Actin cytoskeleton disruption, phagocytosis inhibition, delaying NADPH oxidase complex assembly, regulation of T2SS	Flannagan et al., 2012; Rosales-Reyes et al., 2012a; Rosales-Reyes et al., 2012b
Type IV secretion system	Intracellular survival and replication	Sajjan et al., 2008
Zinc metalloproteases	Degrading extracellular matrix (type IV collagen, fibronectin), neutrophil protease inhibitor, macroglobulin, and antimicrobial peptides	Kooi et al., 2005; Kooi et al., 2006; Kooi and Sokol, 2009

1.2 **Microbial recognition by the innate immune system and host response**

The respiratory tract is the second largest mucosal surface in human body and is constantly exposed to the outer environment. It is anatomically divided into the upper airways, the lower airways (the trachea and bronchi) and alveoli (Patwa and Shah, 2015). The major role of the respiratory system is gas exchange, which occurs at the alveolar-capillary membrane. The blood-air barrier is extremely thin (average thickness of normal lungs, 1.7 μm) to maximise oxygen diffusion (Divertie et al., 1975). The average volume of gas inhaled into a person's lungs (respiratory minute volume) at rest is about 6 litre/min. Inevitably, small particles and microbes are ventilated into the conducting tract with air, which results in the respiratory system being a favourable entrance for pathogens. Thus, in order to provide warm, hydrated and almost sterile air to the peripheral alveoli, the conducting airways are delicately designed with multiple physical and chemical barriers. True threats must be quickly detected and removed, but unnecessary inflammatory responses should be avoided. Hence, the threshold of sensitivity is critical for normal respiratory functions; otherwise the airway is constantly hyper-responsive and chronically inflamed, causing diseases such as asthma and COPD. Hence, apart from mounting effective immune responses, the respiratory tract is tightly regulated to minimise unfavourable tissue damage and maintain homeostasis of the respiratory system.

1.2.1 Cells of the innate immune system in the airway

Individual cells types shape and orchestrate the innate immunity. From trachea to terminal alveoli, the conducting airways are predominantly lined with epithelial cells. The mucosa and, directly beneath, the epithelium compartment also contain professional immune cells, including DCs, macrophages, monocytes, neutrophils and mast cells (Yu et al., 2016). These cells serve a surveillance role and are regulated by airway epithelial cells.

1.2.1.1 The multifaceted roles of airway epithelium as the first line of host defence

As the first cell type that comes into contact with environmental agents, the epithelium performs a central role in surveillance, protection and immune regulation (Whitsett and Alenghat, 2015; Weitnauer et al., 2016). The composition and morphology of epithelia vary from distal to proximal. Ciliated pseudostratified epithelia, goblet cells, undifferentiated columnar cells and basal cells constitute the epithelium in the large airways (0 to 5th division), while in small airways (6th to 23rd division) having the same

composition, ciliated epithelia and goblet cells are gradually replaced by club cells (Crystal et al., 2008). The gas exchange chamber alveoli are mainly composed of alveolar type I and type II cells. Squamous type I cells are thin and in close proximity to endothelial cells of respiratory capillaries, covering approximately 95% of the surface areas (Crapo et al., 1982; Weibel, 2015). Cuboidal type II cells are critical for producing surfactant lipids and proteins, as well as being the precursors of type I cells during repair (Barkauskas et al., 2013).

The epithelium (Figure 1.3) in the conducting airways is coated with a thin layer of mucus produced by goblet cells and submucosal glands cells (Ballard and Inglis, 2004; Ma et al., 2018). Normal mucus is a low viscous yet elastic fluid, containing water (about 98%), mucins, proteins, lipids, salts and cell debris (Fahy and Dickey, 2010). Inhaled particles are trapped by mucus, thereby being transported promptly to the pharynx by coordinated cilia beating (Kilburn, 1968). This process is a critical innate defence mechanism, namely mucociliary clearance (MCC). Defects in MCC caused by mucus hyperproduction will result in mucus plugs and entrapped microbial substances, which eventually result in pathological conditions, such as COPD, asthma and bronchiectasis (Evans et al., 2009; Ramos et al., 2014; Sears et al., 2015). In addition, the mucus layer restricts the direct contact between microbial substances and airway epithelial cells, which prevents unnecessary inflammatory responses.

Beneath the mucus layer, airway epithelium and apical junctional complexes form a physical barrier against potential pathogenic threats, controlling paracellular transport, and establishing cell polarity. This barrier is a key component of respiratory tract homeostasis as it limits the access of inflammatory ligands to subepithelial professional immune cells. Cell-cell adhesion is built up with the apical tight junctions and underlying adherens junctions (Georas and Rezaee, 2014). While the extracellular loops of tight junction proteins bind to their counterparts on the adjacent cells, the cytoplasmic domains are connected to the actin cytoskeleton by Zonula occludens-1 (Hartsock and Nelson, 2008). Tight junctions are positively or negatively regulated by different factors, such as Zonula occludens-1 exchange, TLR ligands, microbial infection (Kojima et al., 2013; Ragupathy et al., 2014; Yu et al., 2010). Adherens junctions contain transmembrane nectins and E-cadherin, the latter binding to catenins. Modulation of β-catenin affects the expression of E-cadherin, and thereby affects the morphology, differentiation of epithelial cells, and barrier integrity.

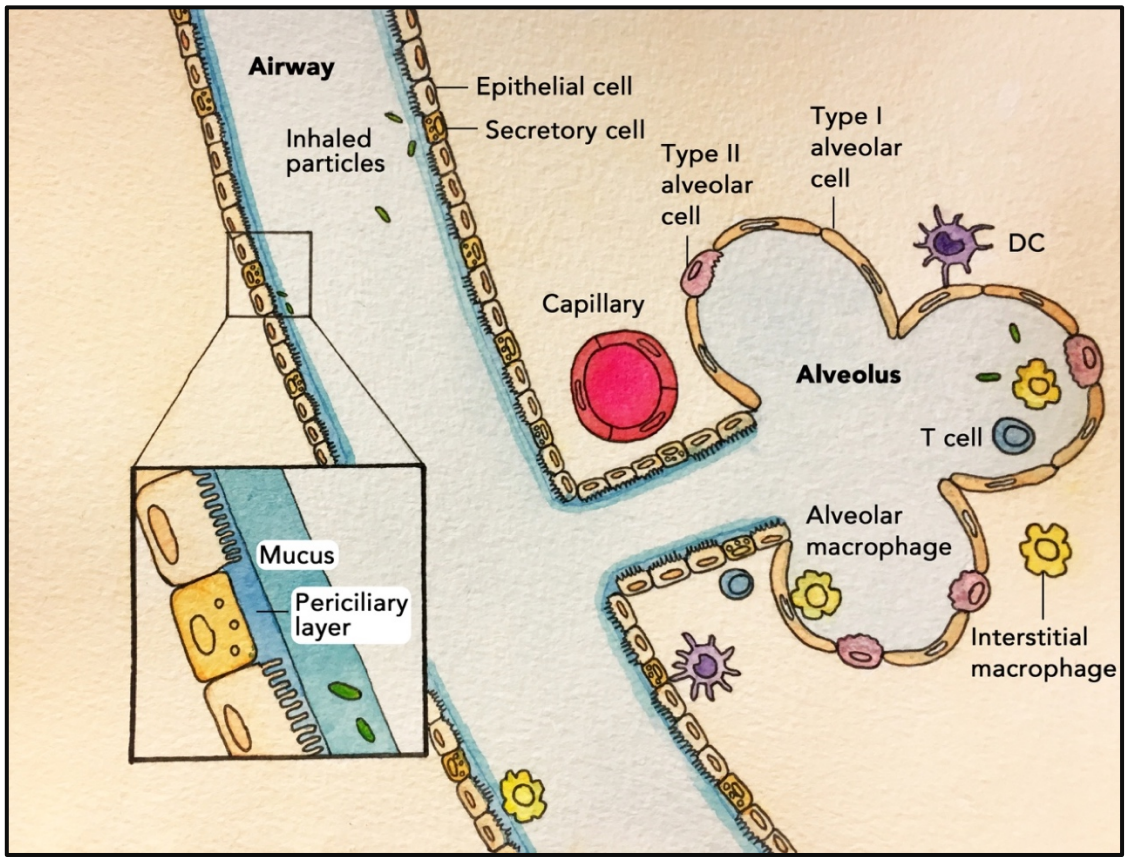

Figure 1.3 Schematic of the conducting airway and alveolus.

The conducting airway is composed of ciliated epithelial cells and secretory cells, while the gas exchange chamber alveolus is composed of type I and II alveolar cells. The airway is lined with a periciliary layer and a mucus layer which are responsible for antigen removal cooperating with cilia beating. The alveolus is coated with surfactant which reduces surface tension and has immunomodulatory effects. Under steady state, professional immune cells such as DCs, T cells, and macrophages reside within the respiratory system or in the interstitial space to perform a surveillance role. Upon activation, macrophages and DCs engulf infectious stimuli and present antigen to T cells. Adapted from Fahy and Dickey (2010), and Hussell and Bell (2014).

Airway epithelial cells, secretory cells, and professional immune cells locally produce RNS, surfactants, antimicrobial peptides, and complement products, which are multi-functional components of host defence. These antimicrobial products are described below.

The free radical nitric oxide has bacterial killing effect in the airway surface fluid at physiologic concentration (Workman et al., 2017). In addition, nitric oxide is a messenger regulating airway smooth muscle relaxation and cilia beating frequency, thereby promoting MCC (Venugopalan et al., 1998; Stout et al., 2007).

Surfactants lipids and proteins are important for normal function and physiology of the respiratory system through reducing alveolar surface tension during respiratory cycle (Whitsett et al., 2010). Moreover, surfactants recognise microbial ligands, C1q, TLRs and their co-receptors, thereby promoting opsonisation and phagocytosis, as well as anti-

inflammatory responses (Ariki et al., 2012; Watford et al., 2001). Alveolar type II cells are the major sources of surfactants; club cells have also been reported to produce surfactants (Calkovska et al., 2015).

Antimicrobial peptides are constitutively produced or induced upon infection. Defensins are small cationic molecules (3-5 kDa) and divided into 2 subgroups, α-defensins and β-defensins (hBDs). In airway epithelial cells, hBD-1 is constitutively expressed and not inducible under inflammatory conditions, whereas hBD-2 expression is only induced by inflammatory stimuli (Kao et al., 2004; Singh et al., 1998). Apart from an antimicrobial effect, hBD-3 is a chemoattractant for macrophage and works synergistically with LL-37 (Abou Alaiwa et al., 2014; García et al., 2001). Furthermore, lysozyme degrades peptidoglycan in Gram-positive bacteria cell wall, and thus is less effective on Gram-negative bacteria (Nash et al., 2006). Lysozyme has synergistic effects with lactoferrin and secretory leucocyte protease inhibitor (SPLI) (Singh et al., 2000). In addition to iron sequestering, lactoferrin impairs biofilm formation, bacterial adhesion, and has immunomodulatory effects (Jahani et al., 2015; Singh, 2004; Siqueiros-Cendon et al., 2014). The antiprotease activity of SPLI protects the epithelium from neutrophil elastase under inflammatory conditions (Hiemstra, 2002).

Lastly, the complement system is activated in response to environmental stimuli in the airway epithelium (Kulkarni et al., 2018). Complement has diverse immunomodulatory functions, including enhancing phagocytosis, connecting the innate and adaptive immunity and regulating homeostasis (Hajishengallis et al., 2017). For example, the level of C3a in sputum isolated from CF individuals is positively correlated with the lung function (Sass et al., 2015). This may due to C3a inhibition on neutrophil recruitment and activity (Shinjyo et al., 2009).

When the amount of foreign antigens in the conducting airways exceeds a certain threshold, to accurately and rapidly recognise invading stimuli, the airway epithelium is equipped with pattern recognition receptors (PRR), which will be described in 1.2.2. Activation of PRR signalling by pathogen associated molecular patterns (PAMP) results in expression of pro-inflammatory cytokines/chemokines, which amplifies the host response such as promoting phagocytosis. Inevitably, antimicrobial mechanisms such as oxidative burst and release of lytic enzymes cause respiratory tissue damage. Therefore, in order to avoid unnecessary damage under homeostatic conditions, the epithelium uses distinct strategies to adjust sensitivity, including secretion of anti-inflammatory molecules, regulating the expression level and asymmetrical expression of PRRs on polarised mucosal barrier.

As a consequence of low or absent expression of TLR2, TLR4, and their co-receptors CD36, CD14, and MD-2, airway epithelial cells fail to initiate a strong pro-inflammatory response to TLR2 and TLR4 ligands (Becker et al., 2000; Ioannidis et al., 2013; Jia et al., 2004; Mayer et al., 2007). The cellular localisation, together with the expression level of TLRs regulate ligand accessibility and tolerance. Asymmetric expression of TLRs on the apical and basolateral surface has been observed in airway epithelium (Ioannidis et al., 2013). Thus, the restricted access to the sensors on the basolateral surface by paracellular junctions only allows incoming pathogens to activate TLRs when they breach the barrier (Humlicek et al., 2007). From bronchi to alveoli, TLR5 expression is shifted from the basolateral to the apical surface, which is correlated with the level of sterility in different parts of the respiratory tract (Shikhagaie et al., 2014). The activation threshold of airway epithelium can be adjusted by pre-exposing to certain stimuli such as respiratory syncytial virus (Monick et al., 2003) or cigarette smoke (Pace et al., 2008), which upregulate the expression and membrane localisation of TLR4, and thereby sensitise airway epithelial cells in response to LPS.

Overall, as a central regulator in host defence, the airway epithelium plays multiple roles to maintain the normal physiology of respiratory system, including barrier function and MCC, secreting antimicrobial and immunomodulatory molecules into the airway surface fluid, surveillance and quickly initiating host defence in cooperation with professional immune cells against infectious agents.

1.2.1.2 The roles of macrophages in host defence against airway infections

Macrophages resident in the lungs at steady state can be divided into interstitial macrophages and alveolar macrophages (Lehnert et al., 1985). Interstitial macrophages can be found in the space between alveolar cells and capillary endothelial cells, while alveolar macrophages are located on the luminal side of alveoli (Figure 1.3). Compared to alveolar macrophages, interstitial macrophages have higher turnover rate, and higher capacity of producing cytokines in response to IFN-γ and LPS (Cai et al., 2014). Interstitial macrophages also contribute to homeostasis by generating IL-10 and inhibiting DCs activity (Bedoret et al., 2009).

Alveolar macrophages serve as sentinels and regulators, due to their phagocytic capacity, expression of PRRs, and ability to generate cytokines and chemokines (Kopf et al., 2015). Under homeostatic conditions, alveolar macrophages mediate the tolerance threshold to harmless exogenous antigens (Thepen et al., 1989). The immunomodulatory factors generated by alveolar macrophages directly suppress T cells, include prostaglandins

(Pica et al., 1996; Ruggeri et al., 2000), nitric oxide (Bingisser et al., 1998), IL-10 (Mittal et al., 2015), and TGF-β (Yoshimura and Muto, 2011). Alveolar macrophages appear to directly inhibit antigen presentation and migration of DCs (Holt et al., 1993; Jakubzick et al., 2006). By competitively engulfing incoming antigens, alveolar macrophages limit the antigen uptake by DCs and thereby block DCs antigen presentation to T cells (Archambaud et al., 2010).

Alveolar macrophages are in close proximity to the alveolar lumen, and hence their activity is tightly regulated by ligands on the surface of epithelial cells (Hussell and Bell, 2014). By cell-cell interactions, latent TGF-β binds to $\alpha_V\beta_6$-integrin on airway epithelial cells and is subsequently transformed into bioactive TGF-β, which suppresses the expression of cytokines in alveolar macrophages (Sheppard, 2006). However, once activated, alveolar macrophages interrupt the cell-cell interactions and thus the brake on cytokine production by TGF-β is released (Morris et al., 2003). In addition, signal-regulatory protein-α, mannose receptor, and CD200 also negatively regulate alveolar macrophages function (Hussell and Bell, 2014; Snelgrove et al., 2008; Zhang et al., 2004).

Once the negative signalling is overridden by infectious agents, alveolar macrophages switch from a tolerogenic mode to inflammatory mode, exhibiting higher phagocytic ability, higher oxidative burst, and stronger expression of pro-inflammatory cytokines. Neutrophils and monocytes are recruited by the signals released from alveolar macrophages (Nomura et al., 2001). To prevent dissemination, alveolar macrophages rapidly engulf apoptotic epithelial cells and apoptotic neutrophils (Rubins, 2003; Grabiec and Hussell, 2016). This process protects the host from toxic products leaked from neutrophils. Once the inflammatory response is unleashed, the outcome depends on the interactions between virulence factors of pathogens and the corresponding host response. Hence, alveolar macrophages serve as a gatekeeper of inflammatory responses in the alveoli.

1.2.1.3 The roles of neutrophils in airway infections

Neutrophils are generated from the bone marrow and circulating in the bloodstream. When infection occurs, airway epithelial cells and alveolar macrophages release inflammatory mediators (such as IL-8, IFNs, C3a, and C5a) and neutrophils respond to the call. Upon interactions between endothelial receptors and the ligands on neutrophils, neutrophils migrate to the site of infection following the concentration gradient of chemoattractants (Thammavongsa et al., 2015).

Once recruited to the battlefield, neutrophils rapidly engulf opsonised bacteria and challenge them with various toxic products in the granules, including antimicrobial

peptides, ROS, nitric oxide, and proteolytic enzymes (van Kessel et al., 2014). Upon phagocytosis, neutrophils initiate apoptosis and release 'eat me' signals (phosphatidylserine) to macrophages, to put a brake on excessive inflammatory responses (Fox et al., 2010). In addition, neutrophils immobilise and kill extracellular bacteria by releasing neutrophil extracellular traps which contains DNA and antimicrobial peptides (Spaan et al., 2013b).

However, the bactericidal activity of neutrophils is equally harmful to the host if out of control. Therefore, in the resolving phase, negative regulators (such as the sialic acid binding Ig-like lectin E, and NADPH oxidase) and macrophage phagocytosis are important to eliminate neutrophils in due course (Davidson et al., 2013; McMillan et al., 2013; Kruger et al., 2015). Prolonged neutrophil presence leads to chronic inflammation, tissue injury, and delayed healing; diseases such as COPD, CF, asthma, and pulmonary fibrosis are associated with the failure to remove apoptotic neutrophils (Hodge et al., 2003; Morimoto et al., 2012; Vandivier et al., 2002).

Overall, neutrophils play a key role in first line defence but require tight regulation to prevent the detrimental effect on the host.

1.2.2 TLR-mediated pathogen recognition and host defence

1.2.2.1 Overview of PRRs

The sentinels of the lower respiratory system, airway epithelial cells and respiratory-resident phagocytes express a wide range of conserved PRRs to detect exogenous PAMPs and endogenous damage-associated molecular patterns, as well as modulate efficient immune responses. PRRs include TLRs, nucleotide-binding oligomerization domain-like receptors (NLRs), C-type lectins, and retinoid acid-inducible gene-I receptors (RLRs) (Takeuchi and Akira, 2010). Located in the cytoplasm, NLRs recognise bacterial ligands (peptidoglycan, flagellin), viral RNA, and fungi, leading to activation of MAPK and NF-κB signalling, inflammasome formation, and type I IFNs production (Kim et al., 2016). C-type lectins binds a wide range of carbohydrates ligands from fungi, bacteria, and viruses (Hoving et al., 2014). Activation of C-type lectins leads to activation of transcription factors, phagocytosis, and the development of T helper 1 and T helper 17 responses (Hardison and Brown, 2012). RLRs recognises intracellular viral double-stranded RNA, following by activation of IFN regulatory factor (IRF)-3 and NF-κB, and ultimately leads to production of IFN-β and antiviral immune responses (Matsumiya and Stafforini, 2010). All PRRs play a key role in sensing exogenous antigens and rapidly initiating the host

response to remove the incoming threats and keeping damage under control. To maintain the homeostasis, PRRs are required to discriminate between self/non-self, commensal/pathogenic antigens, and exhibit tolerance under certain threshold. In the following section, the first discovered PRRs, TLRs, and their interactions with PAMPs will be the focus.

1.2.2.2 Overview of TLRs

The discovery of TLRs in the 1990s was of significant importance to the field of innate immunity and was recognised by awarding the 2011 Nobel Prize in Physiology or Medicine to Bruce Beutler and Jules Hoffmann. TLRs are the key molecules in surveillance, signalling transduction and ultimately induction of immune responses. Their dysfunction is associated with allergic inflammation (Clarke et al., 2014), chronic inflammation, tumorigenesis (Korneev et al., 2017), and autoimmune diseases (Mohammad Hosseini et al., 2015).

So far there have been 10 TLRs (Table 1.3) identified in humans, with each one recognising unique ligands from bacteria, viruses, and fungi. TLRs are widely expressed in leukocytes and non-immune cells (including mucosal epithelial cells, fibroblasts, and endothelial cells), and their expression is regulated by microbial stimuli and cytokines (McClure and Massari, 2014; Zarember and Godowski, 2002). TLR1, 2, 4-6 are primary transmembrane receptors that sense extracellular antigens, whereas TLR3, 7-10 are expressed on the membrane of endoplasmic reticulum (steady-state) and trafficking to endolytic compartments (active-state) to engage with internalised viral ligands. Despite recognising structurally distinct ligands, TLRs share common a architecture. The ligand-binding ectodomain contains leucine-rich repeats, while the cytoplasmic domain contains Toll/IL-1 receptor (TIR) domain for signal transduction (Botos et al., 2011). Upon ligands binding, the extracellular domain dimers form ‘m’ shape which brings the C-terminal of leucine-rich repeats and intracellular domains in close proximity (Botos et al., 2011). TLRs and downstream signalling cascades are bridged by adaptor proteins, including myeloid differentiation primary response 88 (MyD88), TIR domain-containing adapter-inducing IFN-β (TRIF), MyD88 adaptor-like (Mal) (Valkov et al., 2011), TRIF-related adaptor molecule (TRAM), and Sterile-α and HEAT-Armadillo motif-containing protein (Carlsson et al., 2016). All TLRs except TLR3 share MyD88-mediated signalling, whereas TLR3 and TLR4 recruit TRIF; all TLRs lead to activation of MAPK and NF-κB signalling.

Table 1.3 Human TLRs and their exogenous ligands.

TLR	Localisation	PAMPs	Origin of the ligands	Reference
TLR1	Cell surface	Triacyl lipopeptides	Gram-negative bacteria, Mycoplasma	Shimizu et al., 2007
TLR2	Cell surface	Lipoprotein, peptidoglycan, lipoteichoic acid, phospholipomannan	Bacteria, *Mycoplasma*, fungi	Into et al., 2004; Jouault et al., 2003; Schwandner et al., 1999; Love et al., 2010; Muller-Anstett et al., 2010
TLR3	Endolysosome	dsRNA, Poly I:C	Viruses	Alexopoulou et al., 2001
TLR4	Cell surface	LPS	Gram-negative bacteria	Chow et al., 1999
TLR5	Cell surface	Flagellin	Bacteria	Hayashi et al., 2001
TLR6	Cell surface	Diacyl lipoprotein	Gram-positive bacteria	Into et al., 2004
TLR7	Endolysosome	ssRNA, guanosine	Viruses	Diebold et al., 2004; Zhang et al., 2016
TLR8	Endolysosome	ssRNA	Viri, bacteria	Heil et al., 2004; Cervantes et al., 2013
TLR9	Endolysosome	Unmethylated CpG-DNA, chromatin-IgG complexes	Bacteria, viruses	Leadbetter et al., 2002; Rutz et al., 2004
TLR10	Endolysosome	dsRNA	Viruses	Lee et al., 2018

1.2.2.3 TLR-mediated signalling pathways

The adaptor protein for most TLRs, MyD88 plays a central role in TLR-mediated signalling. MyD88 deficiency increases bacterial burden and dampers host defence to *S. aureus* infection (Dudek et al., 2016; Takeuchi et al., 2000; Weiss et al., 2005). Activated TLRs recruit MyD88 by interaction between their TIR domains. As shown in Figure 1.4, MyD88 recruits and activates IL-1 receptor-associated kinase (IRAK) 4, via death domain-death domain association. IRAK4 activates IRAK1/2, leading to autophosphorylation of the IRAKs complex and subsequently the activation of TNF receptor associated factor (TRAF) 6 (Lin et al., 2010). TRAF6 forms a ubiquitin chain with E2 enzyme Ubc13-Uev1A, which promotes polyubiquitination of TRAF6 and inhibitor of nuclear factor κ-B kinase (IKK) γ

(Chen, 2005). Ubiquitinated TRAF6 activates TGF-β-activated kinase (TAK) and TAK1-binding (TAB), whereby the TAK1/TAB complex leads to the activation of the three-tier MAPK module, MAP3K, MAP2K and finally MAPK including stress-related kinase (Erk) 1/2, p38, and c-Jun N-terminal kinase (JNK) (Schröder et al., 2001; Wang et al., 2001; Dhanasekaran et al., 2007). Members of MAPK family control cell proliferation, survival, and differentiation (Li et al., 2003; D'Souza et al., 2008; Khiem et al., 2008). Activation of MAPK facilitates phosphorylation and activation of transcription factor activator protein (AP)-1 (Akira et al., 2006). Activated IKK complex contributes to phosphorylation and degradation of IκBα, depriving its sequestration of p65 NF-κB, and thereby p65 NF-κB translocates into the nucleus and regulates transcription of pro-inflammatory cytokines (Kawai and Akira, 2007). Notably, MAPK and NF-κB exhibit differential activation thresholds in response to TLR4 ligand (Gottschalk et al., 2016). Activation of NF-κB occurs at a much lower dose of lipid A (component of LPS) compared to MAPK. Although activated NF-κB leads to gene expression and macrophage priming, TNF production is only activated in a MAPK-dependent manner. Hence, while NF-κB is responsible for sensing sub-threshold antigens, MAPK tightly controls the sensitivity threshold and production of pro-inflammatory cytokines. In addition to activating MAPK and NF-κB signalling pathways, intracellular TLRs (TLR7, TLR8, and TLR9) regulate type I IFNs production via TRAF6. Upon sensing exogenous nucleic acids sequestered in the endolysosome, recruited MyD88 activates IRAK complex together with TRAF6, followed by activation of IRF7 (Uematsu and Akira, 2007). IRF7 subsequently translocates into the nucleus to mediate type I IFNs expression (Cervantes et al., 2011). The detailed TLR7/8/9-mediated signalling is described by Kawasaki and Kawai (2014).

TLR3 and TLR4 are associated with MyD88-independent pathway and lead to production of type I IFNs and IL-10 (Häcker et al., 2006). Activation of TLR4 via MyD88-dependent signalling results in an early-phase activation of NF-κB. Following TLR4/MD-2/CD14 endocytosis, TRIF and TRAM are recruited to the complex on endolysosome membrane (Fitzgerald et al., 2003; Tanimura et al., 2008). TRAF6 and receptor-interacting protein kinase-1 are recruited to TRIF, resulting in the late-phase activation of NF-κB (Kagan et al., 2008; Weinlich and Green, 2014). Both phases of NF-κB activation are required for pro-inflammatory cytokine production (Kawai and Akira, 2010). TRIF also interacts with non-canonical IKKs TRIF and two protein kinases-binding kinase-1 and IKKε, through TRAF3, which leads to activation of IRF3 and IRF7, as well as production of type I IFNs and IL-10 (Uematsu and Akira, 2007; Häcker et al., 2011; Ullah et al., 2016).

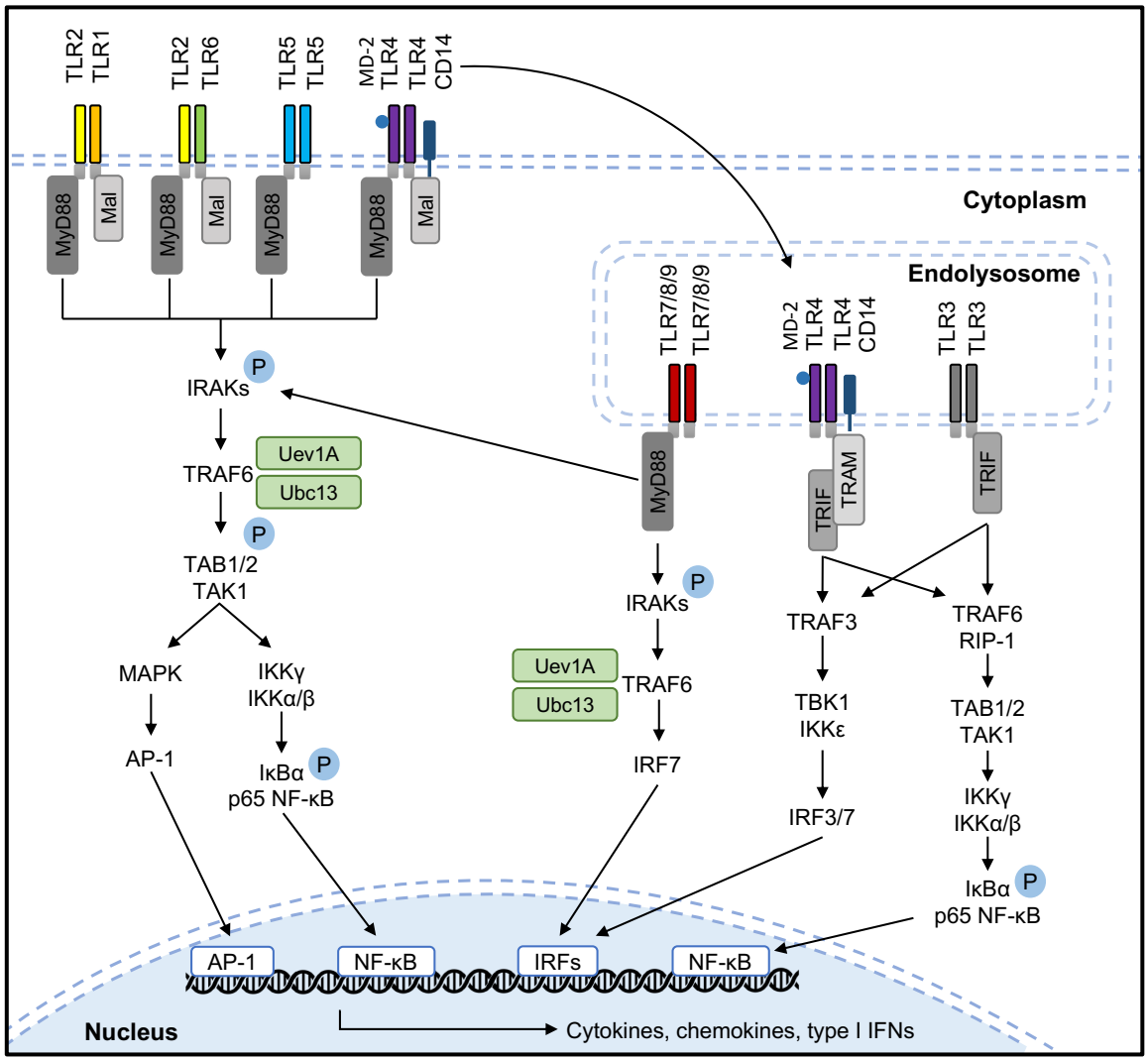

Figure 1.4 MyD88-dependent and MyD88-independent TLR signalling pathways.

The MyD88-dependent TLR signalling pathways mainly result in production of inflammatory cytokines and chemokines, whereas MyD88-independent signalling pathways are responsible for type I IFNs production to promote anti-viral responses. Unlike other TLRs, TLR4 requires both pathways at distinct cellular localization and leads to biphasic activation of NF-κB to induce production of inflammatory cytokines (own original artwork).

1.2.2.4 Function of TLRs

As described above, the main roles of TLRs are sensing infectious stimuli, recognition of self and non-self antigens, and induce the production of cytokines and chemokines. In addition, the outcome of TLR signalling includes modulation of adaptive immunity, and production of antimicrobial peptides.

The activation of TLRs on DCs leads to DC maturation, antigen presentation to antigen-specific T cells, and thereby induces activation and differentiation of T cells (Manicassamy and Pulendran, 2009; Schweighoffer et al., 2017). Hence, the activation of naïve T cells by TLR signalling bridges between innate immunity and adaptive immunity. DC maturation

features increases in major histocompatibility complex, production of costimulatory molecules and pro-inflammatory cytokines. Both MyD88 and TRIF are involved in DC maturation (Bechelli et al., 2016; Hu et al., 2015). The production of type I IFN contributes to cross-priming by DCs (Le Bon et al., 2003). Moreover, TLRs can directly regulate proliferation and cytokine production by T/B cells (Reynolds et al., 2010; Schweighoffer et al., 2017).

TLR-mediated signal transduction leads to production of cytokines (such as IL-1, IL-6, IL-10, IL-13, TNFα, and TGFβ), chemokines (such as IL-8, monocyte chemoattractant protein1, macrophage inflammatory protein 2-α, and chemokine ligand 5), and type I IFNs (mainly IFNα and IFNβ) (McClure and Massari, 2014). These secreted products play an important role in shaping the innate and adaptive immune responses, inflammation, survival, and proliferation (Li et al., 2010). Some products serve as pro-inflammatory regulators (such as IL-1, IL-8, and TNFα), whereas IL-10 and TGFβ suppress the immune response. In addition to inducing antiviral enzymes, type I IFNs modulate DCs, B cells, and T cells function in a direct or indirect manner (Trinchieri, 2010). However, in response to bacterial infection, type I IFNs promote anti-inflammatory responses through suppressing IL-1 production and macrophages (Mayer-Barber et al., 2011).

Induction of antimicrobial peptides by TLR signalling was first reported in drosophila (Tauszig et al., 2000). hBD1-4 (Ryu et al., 2013), inducible nitric oxide synthase (He et al., 2006; Lewis et al., 2011), LL-37 (Rivas-Santiago et al., 2008), and SPLI (Vroling et al., 2011) are inducible in response to TLR agonists.

1.2.2.5 Negative regulation of TLR signalling

Activation of TLR signalling is a key event in innate immunity. However, uncontrolled TLR-mediated pro-inflammatory responses may cause immunopathology and autoimmune disease. For example, expression of TLRs and their responsiveness are increased in macrophages and peripheral blood monocytes from individuals with rheumatoid arthritis, systemic lupus erythematosus, or type 1 and 2 diabetes (Huang and Pope, 2009; Dasu et al., 2010; Devaraj et al., 2008; O'Gorman et al., 2015). Hence, it is necessary to have negative regulation of TLR-mediated responses, at multiple stages including soluble decoy TLR, binding sites overlapping, suppression of signal transduction, and inhibition of transcription (Figure 1.5). The multi-layered negative regulation indicates two possible explanations: first, each negative regulator is indispensable but insufficient to finely tune TLR signalling; second, negative regulators function in different tissue, or at different times (Liew et al., 2005).

Competition of binding sites happens both extracellularly and intracellularly. Extracellular soluble TLR2 and TLR4 have been found in human body fluids and the release is upregulated in response to PAMPs (Iwami et al., 2000; LeBouder et al., 2003; Oever et al., 2014). The expression of soluble TLR requires metalloproteinase that causes the shedding of ectodomain of TLR2 (Langjahr et al., 2014). By competitively binding to TLR2 ligand peptidoglycan as a decoy receptor, soluble TLR2 dampers activation of NF-κB and production of pro-inflammatory cytokines (Iwaki et al., 2002). Furthermore, soluble TLR2 is a biomarker to discriminate inflammatory diseases caused by infectious and non-infectious agents (Oever et al., 2014). The immunomodulatory effect of soluble TLR2 is detailed by Henrick et al. (2016).

Furthermore, TLR signalling can be blocked intracellularly by molecules containing the TIR domain, such as B-cell adaptor for PI3K (BCAP), single immunoglobulin IL-1R-related molecule (SIGIRR), and ST2. By TIR-TIR interaction, BCAP binds to TLR adaptor proteins; phosphorylated BCAP recruits PI3K to cytoplasmic membrane and initiates PI3K/Akt activation, thereby bridging TLR to PI3K/Akt signalling (Ni et al., 2012). There might be 3 mechanisms involved in anti-inflammatory regulation of BCAP/PI3K. First, activated Akt phosphorylation of glycogen synthase kinase (GSK)-3β contributes to production of the anti-inflammatory cytokine IL-10 (Zhang et al., 2016). Furthermore, the transcription factor forkhead box protein O (Foxo) 1 is known to enhance production of pro-inflammatory cytokines in macrophages and airway epithelial cells (Fan et al., 2010a; Seiler et al., 2013). Activation of PI3K/Akt leads to phosphorylation and nucleus exclusion of Foxo1 (Brunet et al., 1999). Lastly, a protein-protein interaction study showed that the association between BCAP and MyD88 can interfere with TIR-domain signalosome and MyD88-IRAKs interaction (Guven-Maiorov et al., 2015). Additionally, PI3K/Akt promotes the pro-inflammatory response by phosphorylating IKK (Bai et al., 2009; Lin et al., 2011); or by phosphorylating GSK-3β, the inhibitor of NF-κB (Cremer et al., 2011). The other two TLR negative regulators SIGIRR and ST2 are transmembrane receptors containing a TIR domain, and hence interfere with TLR- and IL-1R-mediated inflammatory responses, with the exception of ST2 which only disrupts MyD88-dependent signalling (Wald et al., 2003; Brint et al., 2004; Nold-Petry et al., 2015).

Inhibition of signal transduction occurs at multiple stages. IRAK-M is a catalytically inactive kinase expressed in macrophages and monocytes that interferes with TLR signalling at different steps (Kobayashi et al., 2002). Upon stimulation with TLR ligands, IRAK-M blocks dissociation of IRAK1/4 from MyD88, and consequently the formation of IRAKs and TRAF6 (Kobayashi et al., 2002). Nakayama et al. (2004) reported that induction of tolerance by pretreatment with peptidoglycan in macrophages is associated with IRAK-M.

IRAK-M decreases IRAK1 kinase activity, as well as blocking recruitment of IRAK1 to MyD88.

Ubiquitinases and deubiquitinases are associated with post-translational modification in TLR signalling. SOCS1 and SOCS3 have ubiquitin ligase activity (Hovsepian et al., 2013; Yu et al., 2015). Hence, by polyubiquitination, SOCS1 and SOCS3 lead to proteasomal degradation of their targets, including Mal (Mansell et al., 2006), TRAF3/6 (Rothlin et al., 2007; Zhou et al., 2015), and p65 NF-κB (Strebovsky et al., 2011). The anti-inflammatory role by SOCSs is reviewed by Inagaki-Ohara (2014). Another ubiquitin ligase Triad3A causes proteolytic degradation of TLR4 and TLR9 (Chuang and Ulevitch, 2004), as well as TLR adaptors TRIF and Mal (Bibeau-Poirier and Servant, 2008). Overall, ubiquitinases downregulate the level of signal proteins, and thus disrupt or abolish TLR signal transduction. Ubiquitination of TRAF6 is an important step to activate TRAF6 for recruiting and activating TABs/TAK1 complex. Polyubiquitinated TRAF6 can be a substrate for deubiquitinases, such as A20, cylindromatosis D (CYLD), and deubiquitinating enzyme A (DUBA, deubiquitinates TRAF3) (Kayagaki et al., 2007; Bibeau-Poirier and Servant, 2008; Sun, 2010). For example, by removing the ubiquitin chain from TRAF6 and disassociating TRAF6 and Ubc13/UbcH5c, A20 inhibits LPS-induced NF-κB pathway (Shembade et al., 2010).

Detailed TLR endogenous inhibitors are summarised in Liew et al., (2005) and Kondo et al., (2012a). Taken together, negative regulators of TLR signalling contribute to immune homeostasis, protecting the host from overreactive inflammation and yet ensuring sufficient host defence. Individual negative regulators have unique and non-redundant roles as deletion of any individual regulators results in hyperresponsiveness. Although there is overlapping of target proteins, regulators possibly work in a synergistic manner to induce sufficient inhibition. In addition to MyD88-dependent TLR signalling, regulators such as Triad3A and DUBA act via a MyD88-independent pathway. Notably, as TRAFs are adaptor proteins in TNFR family, inhibition of TRAF3 or TRAF6 suppresses TNFR signalling simultaneously (Kondo et al., 2012b). Hence, the pan-inhibition activity of negative regulators connects multiple pro-inflammatory pathways and have a stronger inhibitory effect. Artificial inhibitors of TLR signalling have great potential to treat chronic inflammation and autoimmune diseases; however, so far no TLR antagonists have been approved for clinical treatment (Gao et al., 2017). Furthermore, pathogens have evolved to disturb TLR signalling by producing multiple enzymes, such as kinase, protease, ubiquitinases, and deubiquitinases (McGuire and Arthur, 2015). Thus, it is favourable for pathogens to invade and colonise without initiating host immune responses.

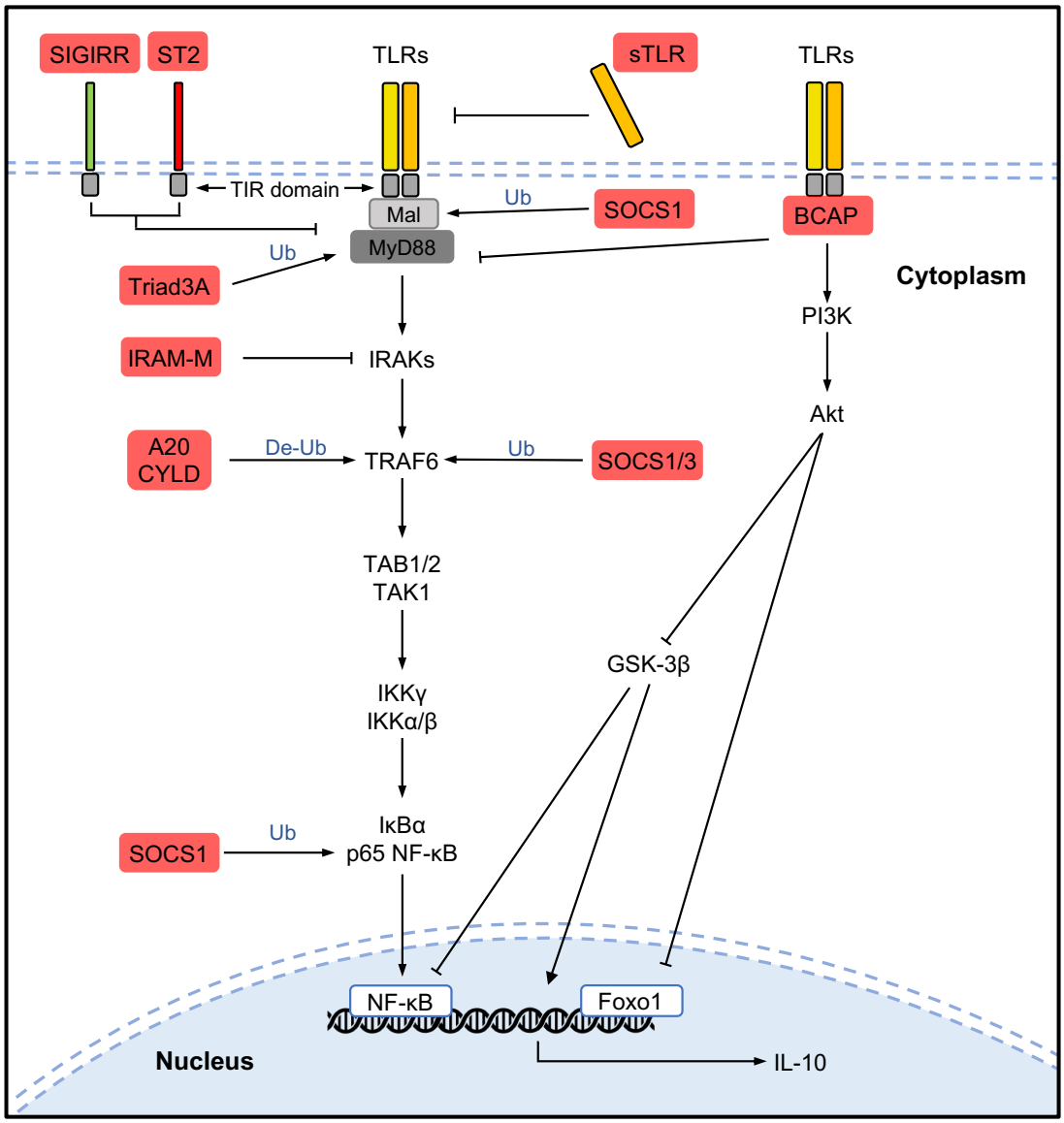

Figure 1.5 Negative regulators of MyD88-dependent TLR signalling pathways.

Negative regulators (in red boxes) play nonredundant roles at distinct steps of TLR signalling. Generated from shedding of TLR ectodomains, sTLRs competitively bind to PAMPs as extracellular decoy receptors. Molecules with TIR domain (BCAP, SIGIRR, and ST2) interferes with TLR/MyD88/Mal complex through competing the binding sites. BCAP also activates PI3K which leads to production of IL-10 and inactivation of transcription factor Foxo1. IRAK-M dampens disassociation of IRAKs and MyD88. Ubiquitinases (SOCS1/3 and Triad3A) and deubiquitinases (A20 and CYLD) disrupt posttranslational modification and thus block signal transduction (own original artwork).

1.3 **Hypothesis and aims**

The introduction above has described host defences and the multiple mechanisms that pathogens have evolved to circumvent these responses.

Bacterial infections in the LRTI mainly exist in polymicrobial communities. Microbial interactions within airway microbiome may alter disease course and clinical outcomes. While Bcc colonisation negatively affects the survival of CF individuals, *S. aureus* infection has the opposite effect and may delay lung disease progression. The underlying mechanism remains largely unknown. It was hypothesised that *S. aureus* interacts with the host defence by manipulating innate signalling pathways, thereby alleviating pathogenesis caused by Bcc virulence. To test this hypothesis, human airway epithelial cells were challenged with *S. aureus* and Bcc single-species infection or co-infection. Immune responses by epithelial cells were recorded and compared.

Aims:

- To assess the modulation of single-species *S. aureus* and Bcc on host signalling cascade and inflammatory responses
- To study the effect of *S. aureus* / *B. cenocepacia* co-infection on host signalling and inflammatory responses
- To interpret the host target of Staphylococcal immunomodulatory factors
- To identify the nature of immunomodulatory molecules released by *S. aureus*

Chapter 2 Material and Methods

2.1 Human Airway Epithelial Cell Lines

Human bronchial epithelial 16HBE14o (16HBE, obtained from Prof Dieter C. Gruenert, University of California San Francisco, US) was isolated from surface epithelium of 2nd division bronchi of a 1-year old male heart-lung transplant patient and transformed by simian vacuolating virus 40 large T-antigen (Cozens et al., 1994). 16HBE cells retain tight junction, cilia structure, and cystic fibrosis transmembrane conductance regulator expression (Cozens et al., 1994). Human airway epithelial Calu-3 was purchased from ATCC (HTB-55). Isolated from the pleural effusion of a 25-year old male with a pulmonary adenocarcinoma, Calu-3 cells display epithelial morphology, barrier integrity and cystic fibrosis transmembrane conductance regulator under defined conditions (Fogh et al., 1977; Kreft et al., 2015).

16HBE cells and Calu-3 cells were maintained in complete medium containing Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS), 100 units/mL penicillin and 100 µg/mL streptomycin (Gibco). Cells were incubated in 75 cm^2 flasks (Nunc) at 37ºC in a humidified atmosphere with 5% CO_2.

For dissociation, cells were rinsed with 5 mL Dulbecco's phosphate buffered saline (DPBS), followed by incubating with 5 mL 0.25% trypsin-EDTA (Gibco) for 10 minutes at 37ºC. After adding 5 mL complete medium to neutralise trypsin, the number of viable cells were determined using trypan blue exclusion. Briefly, 50 µL cell suspension was mixed with same volume trypan blue (0.4%), and loaded on a haemocytometer. Viable cells in the four large squares were counted and cell density (cells/mL) was calculated as: number of viable cells $\times$ 2 $\times$ 10^4 / 4. After centrifugation at 1000 *g* for 5 minutes, cells were resuspended in complete medium and plated out at a density of 5×10^5 cells/well into 12-well plates or 2.5×10^5 cells/well into 24-well plates.

For storage, cell suspensions were adjusted to 4×10^6 cells/mL in freeze medium (50% DMEM, 40% FBS,10% DMSO, v/v), transferred into cryovials and stored in liquid nitrogen.

2.2 Bacteria Strains

The bacterial strains used in this study are listed in Table 2.1. Bacterial cultures were inoculated on Luria-Bertani agar (LB, 1% tryptone, 0.5% yeast extract, 1% NaCl, 1.5% agar-agar, w/v) from -80ºC stocks every week, incubated at 37ºC for 1 day and kept at 4ºC. Due to its slow growing nature, *Burkholderia* strains were incubated at 37ºC for 2 days and maintained at room temperature. A single colony of the strain was transferred to LB broth at 37ºC with shaking for about 16 hours. Overnight cultures were diluted at 1

in 20 and cultured until exponential phase as determined by the growth curve. Bacteria were centrifuged at 5000 *g* for 10 minutes and washed once with sterile PBS (0.01 M phosphate buffer, 0.0027 M KCl and 0.137 M NaCl). The bacterial cell density was adjusted based on optical density (OD) measured at a wavelength of 600 nm to meet different multiplicity of infection (MOI, the ratio of bacteria to cells) values. When tryptone soya broth (TSB) was used, bacteria were cultured in TSB (1.7% pancreatic digest of casein, 0.3% enzymatic digest of soya bean, 0.5% NaCl, 0.25% K_2HPO_4, 0.25% glucose) at 37ºC with shaking for 16 hours.

Heat-killed bacteria were prepared by adjusting the OD_{600} of cultures to the desired MOI and heating these on a heating block at 100ºC for 1 hour. Bacterial supernatants were prepared by centrifuging overnight cultures at 5000 *g* for 10 minutes and filtered through a 0.2 µm membrane. Heat-inactivated supernatants were prepared by heating samples at 95 ºC for 10 minutes. To fractionate excreted products from *S. aureus*, supernatants were submitted to 10-kDa- and 3-kDa-cutoff centrifugal concentrators (Vivaspin 20, Sartorius), centrifuged at 5000 *g* for 17 minutes and 180 minutes respectively following the manufacturer's instructions. Supernatants were separated into three fractions: >10000 molecular weight cut-off (MWCO), 3000-10000 MWCO, and <3000 MWCO. Filtrates and concentrates were adjusted to the original volume with LB, followed by filter sterilisation. Immobilised proteinase K (PK) was reconstituted in Tris-Cl (50 mM, pH 8) / $CaCl_2$ (10 mM) and used at a concentration of 100 µg/mL at 37ºC for 1 hour. To prepare inactivated PK, PK was heated at 99ºC for 1 hour. Immobilised PK mixture was subsequently removed by centrifugation at 250 *g* for 10 minutes. All bacterial products were aliquoted and stored at -80ºC.

For long-term storage of bacterial strains, overnight cultures were transferred to a cryovial with sterile glycerol (final glycerol concentration 25%, v/v) to limit cellular damage, vortexed and stored at -80ºC.

Table 2.1 Bacterial strains used in this study.

Strain	Characteristics	Source	Reference
S. aureus			
MRSA252	Hospital-acquired MRSA, fatal post-op septicemia	E.J.Feil	Holden et al., 2004
MSSA NCTC 6571	Methicillin-sensitive *S. aureus*	NCTC	
MR31	Clinical isolate	R.C. Massey	Trzciński et al., 1997

Table 2.1 (continued)

MR81	Clinical isolate	R.C. Massey	Trzciński et al., 1997
MRSA USA400	Community-associated *S. aureus*	R.C. Massey	Voyich et al., 2006
MRSA41		R.C. Massey	Collins et al., 2010
MSSA C316		R.C. Massey	Robinson et al., 2005
MSSA NCTC 10788		NCTC	
MSSA Newman	Isolated from a secondary osteomyelitis infection of a TB patient	R.C. Massey	Baba et al., 2008
MSSA RN4220	Derivative of NCTC8325-4, with *agr*A mutation	R.C. Massey	Nair et al., 2011
MSSA SH1000	Laboratory strain, derivative of NCTC8325 by complementation of *rsbU*	R.C. Massey	O'Neill, 2010
MSSA160		R.C. Massey	Feil et al., 2003
MSSA209		R.C. Massey	Collins et al., 2010
US119		R.C. Massey	
Bcc			
B. multivorans DSMZ 13243	Isolated from a CF patient	DSMZ	
B. cenocepacia J2315	Isolated from a CF patient, epidemic ET12 lineage	J. R. W. Govan	Holden et al., 2009
B. anthina J2552	Botanical isolate	J. R. W. Govan	Palfreyman et al., 1997
Reference strains			
E. coli HB101	Non-invasive K-12 stain		Boyer and Roulland-Dussoix, 1969
Salmonella Typhimurium NTB6		Radboud collection	Boleij et al., 2011b

2.3 Biofilm Assay

Biofilm formation was assessed as described before (Coffey and Anderson, 2014). Briefly, overnight cultures were diluted 1:100 with LB, and 100 µL aliquots were added to a 96-well microtiter plate. After 24 hours incubation at 37ºC on a 3-dimensional rotator, planktonic bacteria were removed, and the biofilms were gently rinsed with PBS twice. Biofilms were stained with 125 µL of 0.1% (w/v) crystal violet for 10 minutes. Excess crystal violet was removed by washing the plate three times in trays filled with lukewarm tapwater, after which the biofilm was dried in a 60ºC oven. Crystal violet was dissolved with 150 µL of 20% (v/v) acetic acid and 125 µL of each sample was transferred to a new plate. The absorption was read at a wavelength of 550 nm.

2.4 Measurement of Epithelial Barrier Integrity

2.4.1 Measurement of Transepithelial Electric Resistance (TEER)

Calu-3 cells were plated into Transwell permeable supports (12 mm diameter, Corning) at 5×10^5 cells/mL, 0.3 mL per insert. The basolateral chamber was supplied with 1 mL complete medium. Medium in the apical chamber was removed 48 hours after seeding to induce the formation of polarised monolayer with tight junction.

TEER was monitored by EVOM Volt/Ohm Meter and STX2 electrode (World Precision Instrument). STX2 electrode was sterilised in 100% ethanol and washed once with DMEM. As shown in the schematic (Figure 2.1), serum-free DMEM (300 µL) was added to the apical chamber. After 30 minutes incubation, the long electrode was placed into the basolateral chamber and the short electrode in the apical chamber without damaging the monolayer. After subtracting the readout of inserts with medium (background) from raw data, they were normalised for area, giving TEER values in Ohm.cm^2 as in the following equation: (total resistance-background resistance) $\times$ 1.12cm^2. Higher value of resistance indicates higher confluency of cells on the inserts as less ions can transport through the membrane. Calu-3 cells achieved a polarised state within 7 days when the TEER reached >400 ohm.cm^2.

Polarised Calu-3 cells were infected apically with MRSA252 and *B. multivorans* 13243 (0.5 mL) for 24 hours. TEER was recorded at time 0, 3 and 24 hours post infection.

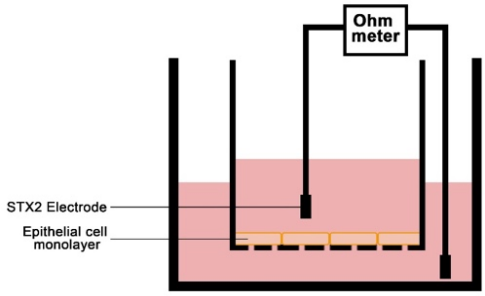

Figure 2.1 Schematic of measuring TEER for airway epithelial cell monolayer.

STX2 electrode is used to measure the TEER. The total readout includes the electrical resistance of the cell monolayer, the membrane and culture medium.

2.4.2 Flux of Fluorescein Isothiocyanate (FITC) Labelled-Dextran

Calu-3 cells were infected with MRSA252 and *B. multivorans* 13243 as described above. At 24 hours postinfection, the inserts were washed twice with Hanks' Balanced Salt Solution (HBSS) gently to remove phenol red. Two-hundred-microlitre FITC-dextran (molecular weight 3000-5000, 500 µg/mL in HBSS) were applied to the apical side and 600 µL HBSS to the basolateral side. After 30 minutes incubation at 37 ºC, medium from the basolateral chamber was collected and fluorescence intensity was determined using a FLUOstar OPTIMA plate reader (BMG Labtech) at 485 nm excitation wavelength and 530 nm emission wavelength. The readout was normalised by subtracting the background (HBSS), and was divided by maximum (the insert without monolayer) to obtain the value of percent epithelial permeability.

2.5 Invasion Assay

To study the role of PI3K/Akt signalling, epithelial cells were pretreated with LY294002 or wortmannin for 30 or 60 minutes until the end of invasion period. 16HBE cells were infected with MRSA252, MSSA NCTC 6571, *B. multivorans* 13243, *B. cenocepacia* J2315, *B. anthina* J2552, *E. coli* HB101 or *S.* Typhimurium NTB6 at MOI 50 for 2 hours. The number of bacteria in the inocula were determined by viable count. 16HBE cells were rinsed three times with sterile DPBS to remove unattached pathogens and lysed with 100 µL saponin (1% w/v in trypsin-EDTA). The lysates were plated on LB agar plates to count the number of bacteria that were attached to the epithelial cell membrane. Alternatively, following 2 hours infection and DPBS rinse, medium was supplemented with antibiotics (Table 2.2) to eliminate extracellular bacteria for the time indicated in Table 2.2. To ensure more than 99.9% bacterial killing effect, the last DPBS wash was plated on LB agar plates as well. Subsequently, epithelial cells were lysed with 100 µL saponin and internalised

bacteria were determined by viable count. Adherence is shown as a percentage of the inoculum, and internalisation as a percentage of adherence.

Table 2.2 Antibiotics treatment for bacterial strains.

Strains	Antibiotics	Time
S. aureus isolates	Lysostaphin (20 μg/mL) + vancomycin (25 μg/mL)	1 h
B. multivorans 13243 *B. anthina* J2552	Ceftazidime (1 mg/mL) + amikacin (1 mg/mL)	2 h
B. cenocepacia J2315	Ciproflaxin (1 mg/mL) + amikacin (1 mg/mL)	2 h
E. coli HB101	Gentamicin (200 μg/mL)	1 h
S. Typhimurium NTB6	Gentamicin (200 μg/mL) + ampicillin (50 μg/mL)	1 h

2.6 Western Blot Analysis

After stimulation, 16HBE or Calu-3 cells were rinsed with cold DPBS twice and lysed with 100 μL RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 25 mM Tris-Cl pH 8.0, 150 mM NaCl), supplemented with PhosSTOP (Roche) and protease inhibitor cocktail (2 mM AEBSF, 0.3 μM aprotinin, 116 μM bestatin, 14 μM E-64, 1 μM leupeptin 1 mM EDTA; Sigma) just before use. Lysates were collected, centrifuged at 13 000 *g* for 10 minutes at 0ºC and the protein concentrations were quantified using Pierce™ BCA Protein Assay Kit (Thermo Scientific) according to the manufacturer's instructions. Briefly, 10 μL of sample was diluted 1 in 10 with Milli-Q water, and 25 μL of diluted sample was pipetted into a 96-well microtiter plate in triplicate and mixed with 200 μL Working Reagent (0.08% cupric sulfate w/v). Standards ranged from 25 to 2000 μg/mL. The plate was covered and incubated at 37ºC for 30 minutes. The absorbance was read at a wavelength of 550 nm.

Protein samples with equivalent total protein concentrations were mixed with 5 × loading buffer (250 mM Tris-Cl pH 6.8, 500 mM dithiothreitol, 10% SDS, 0.25% bromophenol blue, 50% glycerol, 2% β-mercaptoethanol added before use, v/v). After 95ºC heat-treated for 5 minutes, aliquots were resolved on a 10% SDS-PAGE gel at 90 volts for 30 minutes and then increased to 110 volts for about 1 hour (Mini-Protein Tetra Cell System, Bio-Rad). Proteins were transferred to a nitrocellulose membrane at 150 mA for 1.5 hours (Mini Trans-Blot Cell, Bio-Rad). Running buffer is composed of 190 mM glycine, 25 mM Tris base, 0.1% w/v SDS, and transfer buffer is composed of all these chemicals supplemented with 20% v/v methanol.

To minimise nonspecific binding of the primary antibodies, the membrane was blocked with 5% (w/v) skimmed-milk in TBS (50 mM Tris-Cl, 150 mM NaCl, pH 7.5) for 1 hour at room temperature. Membranes were probed with primary antibodies anti phospho-Akt, phospho-GSK-3β, phospho-NF-κB-p65, phospho-p38, phospho-Erk, phospho-IκBα, or IκBα (Cell Signaling Technology), respectively at 4ºC with gentle rotation overnight. For anti phospho-IκBα and IκBα, same amount of proteins was loaded twice on separate gels and probed separately. After washing with 3×TBST (TBS-0.1% v/v Tween-20) and TBS (each wash 5 minutes), the membranes were then probed with the secondary antibodies, IRDye 800 donkey anti-mouse IgG or 680 donkey anti-rabbit IgG (1:10000 in 5% BSA-TBST) for 1 hour. The blots were washed with 3 × TBST and TBS. Quantitative analysis of the signals from each antibody was performed using the Li-Cor Odyssey Clx imaging system. The membranes were subsequently probed with primary antibodies anti Akt, p38, or Erk, respectively (Cell Signaling Technology), and secondary antibodies as previous. Signal intensity was normalised as a percentage of total Akt, p38 or IκBα on the same membrane.

2.7 Detection of Cytokine Production

At the end of stimulation, conditioned medium of 16HBE cells was centrifuged at 5000 *g* for 10 minutes and supernatants were analysed for IL-8 using a commercially available ELISA kit (eBioscience) according to the manufacturer's instructions. Briefly, Maxisorp ELISA plates (Nunc) were coated with 100 µL capture antibody at 4ºC overnight. The wells were washed three times with Wash Buffer (0.05% v/v Tween-20, PBS) and blocked with 200 µL diluent for 1 hour at room temperature. For each assay, human IL-8 standard was reconstituted using 1 mL distilled water and diluted with ELISA Diluent to 250 pg/mL. The standard curve included 8 points by performing serial 2-fold dilutions of the top standard (range, 2-250 pg/mL). One-hundred microliter standards, samples, and ELISA Diluent (blank) were added to the plate in duplicate. After 2 hours incubation, the wells were washed as previous. The wells were incubated with 100 µL detection antibody for 1 hour, followed by 3 washes. After 30 minutes incubation with 100 µL Avidin-HRP, the wells were rinsed 5 times. One-hundred microliter TMB Solution was added to the wells until 15 minutes later the reaction was stopped by adding 50 µL Stop Solution (2N H_2SO_4). The optical density was read at 450 nm and 550 nm, using a FLUOstar OPTIMA (BMG Labtech) microplate reader. The values of 550 nm were subtracted from those of 450 nm; the values of the blank wells were subtracted from those of standards and samples. Standard curve was generated by graphing concentration of IL-8 versus optical density and used to determine the concentration of IL-8 in the samples.

2.8 Cell Viability Assay

To confirm that the modulation of IL-8 production by heat-killed *S. aureus* or the supernatants was not due to cell damage, epithelial metabolic activity was assessed using the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). 16HBE cells were plated in 96-well plates (5000 cells/well) and incubated for 24 hours. At 24 hours post-stimulation with 10% (v/v) *S. aureus* supernatants or heat-killed *S. aureus*, cells were cultured with 12 mM MTT-DMEM for 2 hours. Formazan formed from MTT in viable cells was dissolved in DMSO for 10 minutes before the optical density was read at 550 nm wavelength.

To determine cell damage caused by live bacteria, 16HBE cells were stimulated with *S. aureus* strains (MOI 0.5) and *B. cenocepacia* J2315 (MOI 5). 16HBE were seeded at 2×10^4 cells/well in 96-well plates one day before experiments (0.1 mL DPBS into the peripheral wells of the 96-well plates). At 2 hours postinfection, cells were rinsed with DPBS twice and then incubated for 24 hours in 0.1 mL phenol red-free DMEM to minimise background absorbance. Lactate dehydrogenase (LDH) release was measured with an LDH Cytotoxicity Assay Kit according to manufacturer's instructions (Pierce). Briefly, 45 minutes before collecting supernatants, 10 µL sterile water was added to one set of triplicate wells as spontaneous LDH activity control; 10 µL Lysis Buffer was added to another set of triplicate wells as maximum LDH activity control. After incubated at 37ºC for 45 minutes, 50 µL supernatants were mixed with 50 µL reaction mixture in a new 96-well plate and incubated at room temperature for 30 minutes in the dark. The reaction was stopped by adding 50 µL Stop Solution. The absorbance was read at 450 nm and 600 nm, using a FLUOstar OPTIMA (BMG Labtech) microplate reader. The 600 nm absorbance value was subtracted from the 450 nm absorbance value. Background control value (DMEM with no cell) was subtracted from average of each sets of readouts and calculated %Cytotoxicity using the following equation.

%Cytotoxicity=100×(samples value-spontaneous LDH activity control)/maximum LDH activity control

2.9 Statistical Analysis

Data were expressed as the mean ± standard error of the mean (SEM) n≥3 independent biological repeats. Results were compared using one-way or two-way ANOVA, followed by Tukey or Dunnet multiple comparisons test, with $p<0.05$ considered significant. The Pearson correlation score with two-tailed test of significance was used for rank correlations.

2.10 Reagents and Consumables

Table 2.3 Reagents and consumables for mammalian cell and bacteria culture.

Product	Supplier	Catalog number
Reagents and consumables for cell culture		
12-well clear sterile plate	Thermo, UK	150628
24-well clear sterile plate	Corning, UK	3527
96-well black plate	Greiner Bio-one, UK	655087
96-well clear sterile plate	Corning, UK	CLS3595
DMEM	Gibco, UK	41966052
DPBS	Gibco, UK	14190250
FBS	Gibco, UK	10082147
HBSS	Gibco, UK	14025092
Penicillin-Streptomycin	Gibco, UK	15140122
T75 cell culture flasks	Nunc, UK	156499
Transwell cell culture inserts	Sigma, UK	CLS3460
Trypsin-EDTA (0.25%)	Gibco, UK	25200072
Tweezer electrode	World Precision Instrument	STX2
Reagents and consumables for bacterial cultures		
Agar-agar	Fisher, UK	10548030
Immobilised proteinase K	Sigma, UK	82452
LB broth, Miller	Fisher, UK	BP1426
PBS tablets	Sigma, UK	P4417
Tryptone soya broth	Oxoid, UK	CM0129
Vivaspin, 10000 MWCO	Sartorius, UK	VS2001
Vivaspin, 3000 MWCO	Sartorius, UK	VS2091

Table 2.4 Drugs for *in vitro* treatment.

Product	Supplier	Catalog Number
Flagellin, FLA-ST	Invivogen, UK	tlrl-stfla
hTLR5-Fc	Invivogen, UK	fc-htlr5
Lipopolysaccharides	Sigma, UK	L2880
LPS-RS	Invivogen, UK	tlrl-rslps
LY294002	Sigma, UK	L9908
Pam3CSK4	Invivogen, UK	tlrl-pms
Recombinant human IL-1β	Peprotech, UK	200-01B
Wortmannin	Sigma, UK	W1628

Table 2.5 Reagents for western blot.

Product	Supplier	Catalog Number
2-mercaptoethanol	Sigma, UK	M3148
30% acrylamide/bis	Bio-rad, UK	1610154
Ammonium persulfate	Fisher, UK	10744171
Glycine	Fisher, UK	10080160
Prestained protein standards	Bio-rad, UK	1610375
Protease inhibitor cocktail	Sigma, UK	P2714
Roche PhosSTOP tablets	Sigma, UK	4906845001
SDS	Sigma, UK	L3771
TEMED	Fisher, UK	10549960
Tris base	Sigma, UK	252859

Table 2.6 List of primary antibodies.

Antigen target	Product Number[a]	Dilution	Diluent	Host
Akt	2920	1:2000	5% skimmed-milk-TBST	Mouse
Erk1/2	4695	1:1000	5% BSA-TBST	Rabbit
IκBα	4814	1:1000	5% skimmed-milk-TBST	Mouse
p38	8690	1:1000	5% BSA-TBST	Rabbit
Phospho-Akt (S473)	4060	1:2000	5% BSA-TBST	Rabbit
Phospho-Akt (T308)	13038	1:1000	5% skimmed-milk-TBST	Rabbit
Phospho-Erk1/2	9106	1:2000	5% skimmed-milk-TBST	Mouse
Phospho-GSK-3β	5558	1:1000	5% BSA-TBST	Rabbit
Phospho-IκBα	9246	1:1000	5% skimmed-milk-TBST	Mouse
Phospho-p38 (T180/Y182)	9216	1:2000	5% skimmed-milk-TBST	Mouse
Phospho-p65	3033	1:1000	5% BSA-TBST	Rabbit

[a] All the products were purchased from Cell Signaling Technology, MA, US

Table 2.7 List of secondary antibodies.

Product	Product Number	Supplier
IRDye 800CW donkey anti-mouse	92632212	Li-Cor, UK
IRDye 680RD donkey anti-rabbit	92668073	Li-Cor, UK

Table 2.8 Assay kits.

Product	Product Number	Supplier
IL-8 human ELISA kit	eBioscience, US	88-8086-88
Pierce BCA protein assay kit	Thermo, UK	23225
Pierce LDH cytotoxicity assay kit	Fisher, UK	13454269

Chapter 3 Investigating Host Response to Bacterial Infections in Human Airway Epithelial Cells

3.1 Introduction

The lung is the major organ of the respiratory system in humans. It is exposed to inhaled microorganisms and particles continuously. To resist infectious attack, the lung has developed sophisticated defence mechanisms. The physical barrier which comprises of airway epithelial cells and the mucus layer plays a major role in limiting entry and assisting clearance of pathogens. The coordinated beating of the cilia on the apical surface of epithelial cells enables removal of particles that are trapped by mucus (Bustamante-Marin and Ostrowski, 2017). Paracellular junctional complexes play an essential role in controlling the transport of water, ions and immune cells, and separating airborne pathogens from the interior of the body to maintain homeostasis in the lung (Brune et al., 2015). However, the epithelial barrier is compromised when the traversal of pathogens occurs (Kim et al., 2005; Malik et al., 2015). To study the barrier function, cells can be cultured on a porous membrane, which is the most common method to represent the morphology and tight junctions of airway epithelial cells *in vitro* (Grainger et al., 2006). In this it is important that the cells used do polarise and form tight junctions as cells would normally do *in vivo*; not many pulmonary cell lines do, but the Calu-3 cell line is one of the few exceptions (Shen et al., 1994). When a confluent monolayer is achieved, the apical side is separated from the basolateral membrane and therefore displays a certain TEER value, which is a measure of the integrity of airway epithelial. This integrity may be compromised by bacteria, but so far little work has been done on effect of MRSA and Bcc, two major lung infection species.

Airway epithelial cells also serve as a sentinel to detect intruding pathogens through a wide range of PRRs (Takeuchi and Akira, 2010). Activation of PRRs lead to a sequence of events, including activation of downstream signalling pathways, initiation of the expression of proinflammatory cytokines, and recruitment and activation of immune cells to the site of infection. Eventually pathogens are resolved by phagocytosis, or antimicrobial substances released by professional phagocytes if the microbial clearance is successful (Nagl et al., 2002). In this process, IL-8 plays an important role at the early stage of infection by chemoattracting granulocytes such as neutrophils. Hence, the level of IL-8 is a biomarker of pro-inflammatory responses and inflammation.

The PI3K/Akt pathway is one of the downstream signalling cascades activated by TLRs, regulating a wide range of cellular functions, such as survival, proliferation and the inflammatory response (Hawkins and Stephens, 2015; Rosadini and Kagan, 2017). For example, Akt is required for the proinflammatory response to *B. cenocepacia* in human peripheral blood mononuclear cells (PBMC) using a PI3K inhibitor (Cremer et al., 2011).

In contrast, inhibition of PI3K/Akt signalling abolished anti-inflammatory cytokine IL-10 production but did not affect proinflammatory cytokine TNFα production by PBMCs in response to *S. aureus* isolates (Peres et al., 2015). Therefore, the discrepancy of PI3K/Akt signalling might indicate that it has pleiotropic roles in regulating inflammatory response, depending on the stimulus and other signals generated.

S. aureus and Bcc are the leading cause of lower respiratory tract infections. Despite being a commensal microbe in the respiratory tract, *S. aureus* is able to colonise the host in a pathogenic manner, which leads to pneumonia with an intense and exaggerated host response. The major challenge of *S. aureus* infection is rapidly evolved antibiotic resistance (Foster, 2017). After acquisition of the resistance gene *SCCmec*, a subgroup of *S. aureus* becomes resistant to most β-lactam antibiotics, namely MRSA. The incidence of individuals with CF harbouring MRSA increased from 5% to 26% from 2000 to 2010, and this has plateaued ever since (Cystic Fibrosis Foundation, 2017). On the other hand, the prevalence of Bcc is about 3% among persons with CF. The acquisition of *Burkholderia* infections are most likely from the natural environment such as soil, and some epidemic strains are well adapted to infectious disease and have greater ability for interpatient transmission (Govan et al., 1993; LiPuma et al., 2002). While some patients have no deterioration, about 20% of infected patients develop cepacia syndrome either soon after the first positive isolate or many years after (Blackburn et al., 2004). Cepacia syndrome is a fatal illness characterised as rapid deterioration of pulmonary function, bacteremia and sepsis. Pre-operative Bcc colonisation is also a significant marker for post lung transplantation mortality (De Soyza et al., 2010). The difficulty of treating Bcc is recognised with their intrinsic and acquired antibiotic resistance. Multi-antibiotic resistance is observed in *Burkholderia* species due to the expression of β-lactamase, efflux pump and outer membrane permeability barrier (Fraser-Pitt et al., 2016; Rhodes and Schweizer, 2016).

In chronic lung infections, microbial pathogens tend to form so-called biofilms to promote long-term survival under the stress coming from antibiotics treatment and the immune clearance (Archer et al., 2011). These biofilms are sessile communities of bacteria embedded within a self-produced matrix on biotic or abiotic surfaces. Biofilm extracellular polymeric substances comprise polysaccharides, proteins, glycoproteins, glycolipids, and extracellular DNA. Bacteria in biofilm displays altered phenotypes with respect to gene expression, protein production and growth rate (Donlan and Costerton, 2002). By adapting to the sessile type of life, microorganisms in biofilm benefit from several advantages over their planktonic counterparts. The majority of cells in biofilm communities are dormant due to the lack of oxygen and nutrients. Hence, the low metabolic rate and

quiescent state downregulate the susceptibility to antimicrobial agents that inhibit synthesis in the process of cell division (Høiby et al., 2010). In addition, the diffusion barrier in biofilms plays a critical role to reduce the penetration of antimicrobial factors. Indeed, antimicrobial agents can be inactivated or absorbed by the matrix layer before reaching the inner layer of bacteria. For example, a study proved that the polysaccharide Poly-β-1,6-N-acetyl-D-glucosamine in the biofilm matrix restricts fluid transport and sequesters the biocide cetylpyridinium chloride within biofilms of *Actinobacillus pleuropneumonia*e and *S. epidermidis* (Ganeshnarayan et al., 2009). In the final stage of the life cycle of a biofilm, bacteria can detach from the community and disseminate to uninfected sites. Biofilm dispersal is regulated by environmental factors, such as shear stress, and biological signalling. It is a vital step between the persistence and dissemination of bacterial pathogens (Guilhen et al., 2017). Overall, biofilm formation represents a key virulence factor of microorganisms.

This chapter was aimed at investigating the host response to bacterial pathogens in airway epithelial cell lines. First, the disruptive effect on *in vitro* epithelial monolayer model by *S. aureus* and *B. multivorans* were characterised. One of the major virulence factors, biofilm formation, was compared between *S. aureus* and Bcc. Finally, the role of PI3K in the production of the neutrophil chemoattractant IL-8 in response to *S. aureus* and Bcc was assessed by the use of PI3K inhibitors.

3.2 Airway Barrier Function Is Compromised by Bacterial Pathogens

Bacterial invasion compromises the barrier integrity of the tight junctions and epithelial cell death. To assess the integrity of infected Calu-3 monolayers, TEER and FITC-dextran flux were monitored.

TEER levels did not change in the first 3 h infection either with MRSA252 or *B. multivorans* 13243, but a pronounced drop was achieved at 24 h. A difference was observed between MRSA252 and *B. multivorans*. At 24 h, a CFU-dependent decrease was monitored in MRSA252-treated groups, whereas *B. multivorans*-treated monolayers showed the same level of reduction at all dose levels examined (Figure 3.1A and B).

To further assess airway epithelial barrier compromise, the diffusion of FITC-dextran (4 kDa) from the apical to basolateral chamber was measured at 24 h postinfection. FITC-dextran detected from the basolateral chamber showed a dose-dependent increase induced by both strains, and *B. multivorans*-infected monolayers showed greater flux than MRSA252.

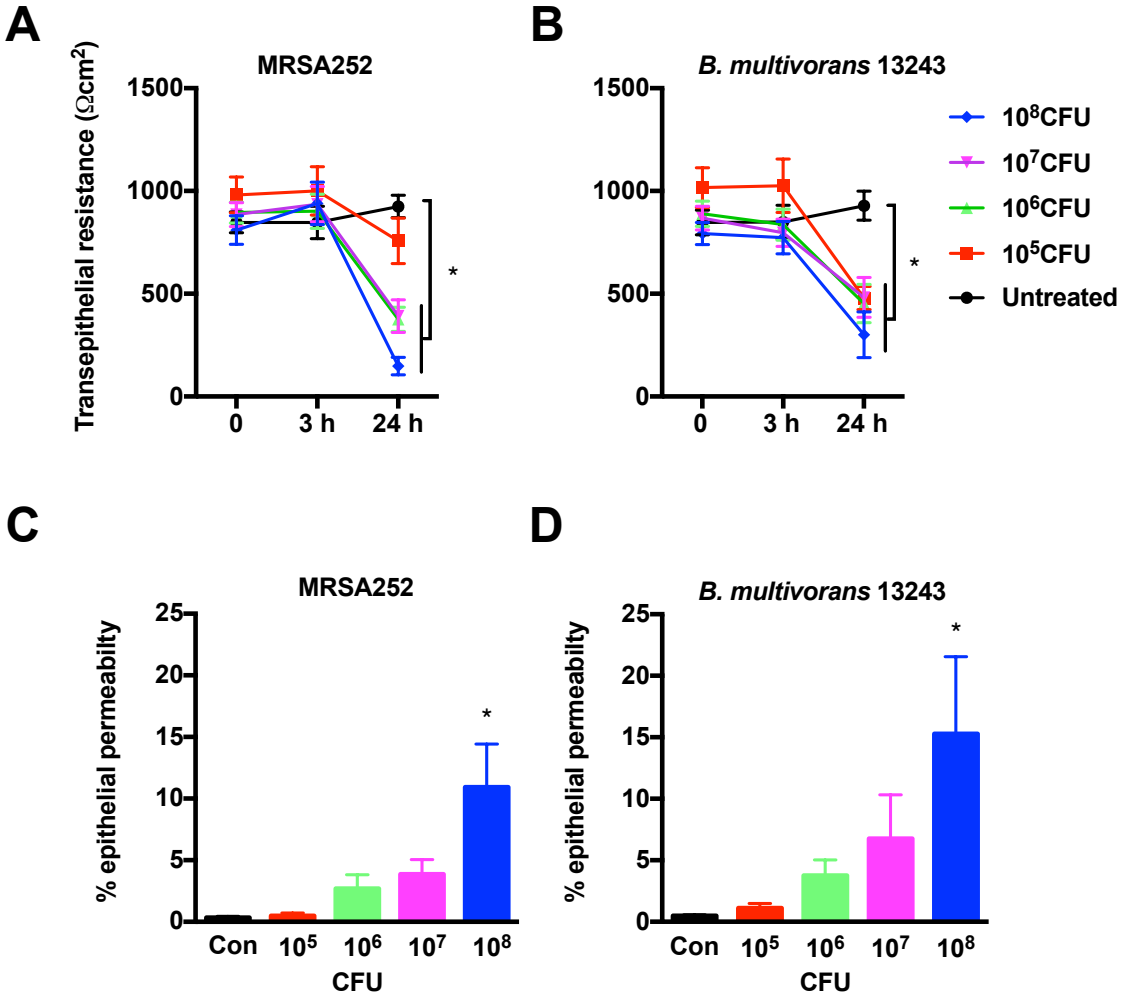

Figure 3.1 Compromised Calu-3 barrier by MRSA252 or *B. multivorans* 13243 invasion.

Polarised Calu-3 monolayers were exposed to increasing CFU of MRSA252 and *B. multivorans* 13243 (0.5 mL apically). **(A and B)** TEER levels were recorded at 3 and 24 h post infection. N=3 independent experiments, performed in duplicate. Error bars indicate mean ± SEM. Data were analysed using two-way ANOVA with Dunnett's multiple comparisons test between untreated and infected monolayers. *, $p< 0.05$. **(C and D)** Diffusion of FITC-dextran through compromised monolayers at 24 h. Data are presented as percent of maximum diffusion (inserts with no cells) and analysed by one-way ANOVA with Dunnett's multiple comparisons test. Compared to untreated monolayers. *, $p<0.05$.

3.3 Evaluation of Biofilm Formation by Bcc and *S. aureus* sp.

To assess biofilm formation of Bcc and *S. aureus* isolates, crystal violet staining was used (Figure 3.2). Cystic fibrosis-isolated strains *B. multivorans* 13243 (absorbance readings of 1.35 ± 0.26) and *B. cenocepacia* J2315 (0.60 ± 0.09) formed abundant biofilms compared to the environmental isolate *B. anthina* J2552 (0.10 ± 0.03). *S. aureus* strains formed same levels of biofilm (0.92 ± 0.42) except MSSA SH1000 (2.80 ± 0.23), which formed 2 times more biofilm than the average biofilm formation. Notably, SH1000 and RN4220 share the same ancestral strain NCTC 8325, but that RN4220 only produced half the amount of biofilm as compared to SH1000. Strain RN4220 differs from SH1000 with defective *agrA* and *rsbU* genes, which are important virulence regulators (Traber and Novick, 2006; Bæk et al., 2013). Compared to RN4220, SH1000 has stronger haemolytic and proteolytic activity, and produces golden pigmentation (Bæk et al., 2013). Overall, heterogenicity of biofilm formation ability was seen between Bcc and *S. aureus* isolates.

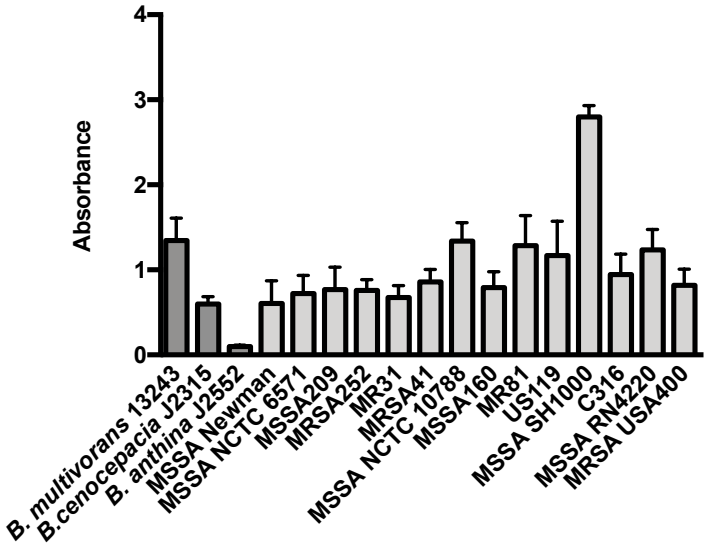

Figure 3.2 Biofilm formation by Bcc and *S. aureus* strains.

Overnight bacterial cultures were diluted, plated into 96-well polystyrene plates, and incubated at 37ºC on a 3-dimentional rotator for 24 h. Biofilm structure was quantified by staining with crystal violet and absorbance read at 550 nm. Data are presented as mean ± SEM from three independent experiments.

3.4 Modification of IL-8 Production by *B. cenocepacia* and *S. aureus* sp. in 16HBE Cells

To study the effect of bacterial pathogens on IL-8 production, 16HBE cells were infected with *B. cenocepacia* J2315 (MOI 5) and *S. aureus* strains (MOI 0.5) for 24 h. IL-8 concentration in the culture medium was determined using ELISA. Under basal conditions 16HBE cells produced low level of IL-8, indicating it has endogenous IL-8 expression (Figure 3.3A). CF-isolated pathogen *B. cenocepacia* J2315 induced a greater level of IL-8 (increased by 102%, $p<0.05$) compared to the baseline. Interestingly, *S. aureus* isolates possessed different abilities of inducing IL-8 in 16HBE cells. Strains such as Newman, NCTC 6571 and MRSA252 induced much less IL-8, whereas some strains stimulated abundant levels of IL-8 (SH1000, C316, RN4220, and USA400, $p<0.05$). However, bacterial pathogens cause damage to epithelial cells, which could potentially result in reduced IL-8 accumulation. To eliminate this possibility, the LDH assay was used to assess cytotoxicity of *B. cenocepacia* J2315 and *S. aureus* isolates (Figure 3.3B). Under basal conditions, a minimal amount of LDH was released (1.9 ± 1.1 %). Even though the inoculum CFU of *B. cenocepacia* J2315 was 10 times higher than *S. aureus*, it caused the lowest level of cell damage (11 ± 1.6 %). This might due to the slow growing nature of J2315 (Kaza et al., 2010), which results in minimal amount of toxins secretion. Among the cytotoxic *S. aureus* isolates, Newman and NCTC 10788 were isolated from a human lesion, MRSA41 is hospital-acquired MRSA, while C316 and USA400 are community-associated *S. aureus*. Most *S. aureus* strains that significantly reduced IL-8 production did not show major cytotoxicity, indicating that the inhibition of IL-8 production was not due to cell death. As biofilm formation is a key virulence factor, we compared IL-8 production in response to *B. cenocepacia* J2315 or *S. aureus* isolates versus biofilm development *in vitro* (Figure 3.3C). However, no correlation was observed between these two variables ($r^2=0.06689$, $p=0.3520$). Taken together, *S. aureus* species show heterogeneity in induction of IL-8 in 16HBE cells, and it is not correlated with biofilm formation.

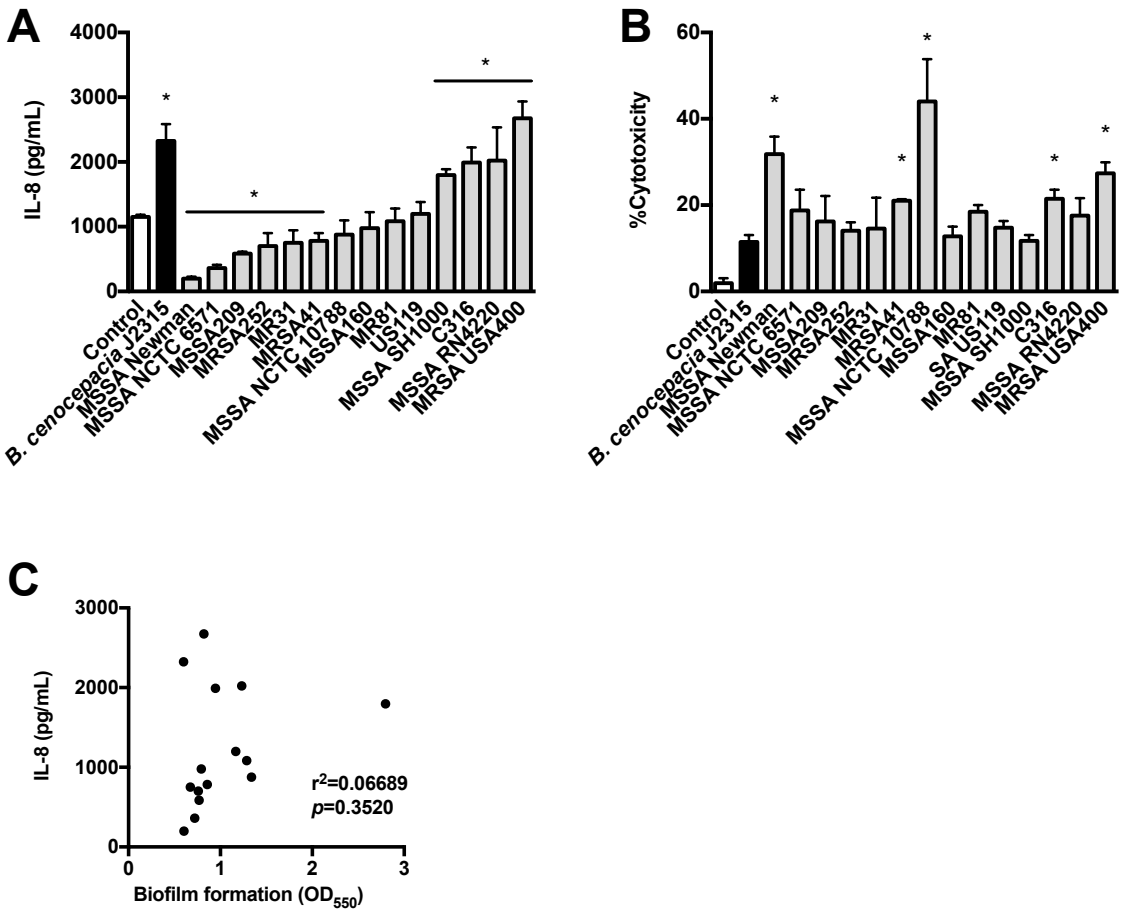

Figure 3.3 IL-8 production and cytotoxicity caused by *B. cenocepacia* J2315 and *S. aureus* sp. in 16HBE cells.

(A) IL-8 secretion in response to *B. cenocepacia* J2315 (MOI 5) and *S. aureus* sp. (MOI 0.5) for 24 h. Data are presented as mean ± SEM from three independent experiments in duplicate. Statistical analysis was performed using two-way ANOVA with Dunnett's multiple comparisons test. **(B)** Cytotoxicity of *B. cenocepacia* J2315 (MOI 5) and *S. aureus* sp. (MOI 0.5) by measuring LDH release. Data are plotted as mean ± SEM from three independent experiments performed in triplicate. Statistical analysis was performed using one-way ANOVA with Dunnett's multiple comparisons test. *, $p<0.05$ compared to the control. **(C)** Scatter plot of IL-8 production in response to *B. cenocepacia* J2315 or *S. aureus* isolates versus biofilm formation. Correlation between biofilm formation and IL-8 was calculated using Pearson correlation score.

3.5 PI3K is required for the proinflammatory response to *B. cenocepacia* J2315

To define the role of PI3K/Akt on host response to *S. aureus* or Bcc infections, 16HBE cells were pretreated with LY294002 or wortmannin for 1 hour and infected with *S. aureus* or Bcc at an MOI of 50. IL-8 concentration in the cultured medium was quantified using ELISA. A heterogeneity was seen in Bcc-induced IL-8 production in the absence of PI3K inhibitors (Figure 3.4). Despite *B. multivorans* 13243 and *B. cenocepacia* J2315 isolated from CF individuals, J2315-infected 16HBE cells produced threefold more IL-8 compared to 13243-infected. As expected, the chemokine response to botanical isolate *B. anthina* J2552 was the lowest, which was consistent with biofilm formation. A slightly higher concentration of IL-8 was seen in response to MRSA252 in comparison with MSSA NCTC 6571. Little or no reduction was seen with the presence of LY294002 or wortmannin, with the exception of J2315. In consistence with Cremer et al (2011), pretreatment with LY294002 or wortmannin caused significant reduction in the J2315-induced IL-8 response (decreased by 34% and 26%, respectively). Thus, these results indicate that the proinflammatory response to J2315 requires PI3K activity in 16HBE cells.

Due to its strong ability of inducing pro-inflammatory chemokine, *B. cenocepacia* J2315 was chosen to investigate pro-inflammatory responses and modulation on host signalling for future experiments.

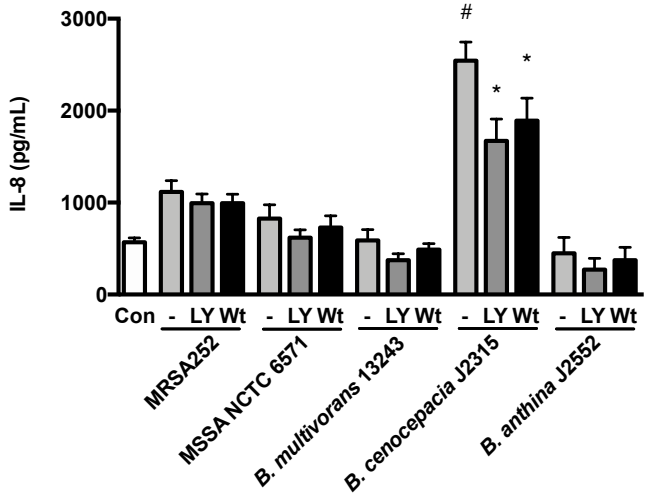

Figure 3.4 IL-8 response to *S. aureus* and Bcc with the presence or absence of PI3K inhibitors in 16HBE cells.

Cells were treated with LY294002 (30 μM) or wortmannin (100 nM) for 1 hour before infected with *S. aureus* or Bcc at an MOI of 50. At 2 h postinfection,16HBE cells were rinsed with DPBS twice and incubated in fresh DMEM for 18 h. IL-8 production was quantified by ELISA. Data are presented as mean ± SEM from three independent experiments performed in duplicate. *, $p<0.05$ compared to J2315-infected cells without any PI3K inhibitors; #, $p<0.05$ compared to the control; using two-way ANOVA followed by Dunnett's multiple comparisons test.

3.6 Discussion

In this chapter, we investigated the host response to Bcc and *S. aureus* isolates, respectively, including the disruption of the airway barrier, induction of pro-inflammatory chemokine IL-8, and cell damage.

Primary airway epithelial cells and cell lines are widely used in host-pathogen interaction studies. Culturing Calu-3 cells on a permeable membrane has proven to represent a great similarity to primary human bronchial epithelial cells phenotype, with cilia-like structures, secretory granules and a high TEER (Grainger et al., 2006). Human bronchial epithelial cells are considered to be the most physiological model, but they have a highly differentiated phenotype due to donor variation, experiment and passage. Calu-3 cells have a more reproducible TEER and phenotype, although data have shown some distinctive differences, such as ZO-1 (a tight junction marker) expression and β-tubulin IV (a ciliated cell marker, expression localised to the cell boundaries) localisation (Stewart et al., 2012). The modulation of MRSA252 and *B. multivorans* 13243 on TEER value and FITC-dextran diffusion of Calu-3 monolayers proves the disruptive effect on the integrity of airway epithelial monolayers. Studies working on Calu-3 cells show that *S. aureus* isolates and *B. multivorans* 13243 caused large falls in TEER (Kiedrowski et al., 2016; Duff et al., 2006). Furthermore, *S. aureus*-secreted products are potent at breaching tight junctions of epithelial barriers (Malik et al., 2015). Duff et al. (2006) demonstrate that although all the tested Bcc isolates reduced TEER within 4 h, translocation speed of Bcc from the apical to the basolateral chamber differs. In order to study the translocation in our model, we cultured Calu-3 cells on 3 μm pore size polyester membrane to allow pathogens to pass through. Despite exhibiting a high TEER value (~600 Ohm.cm^2), Calu-3 cells formed 'leaky' monolayers on 3 μm membranes compared with 0.4 μm filter cultures. Therefore, filter pore size can affect cell morphology and cell densities. In a preliminary experiment, translocation was tested with MRSA252, MSSA NCTC 6571, *B. multivorans* 13243, *E. coli* HB101 and *S.* Typhimurium NTB6 at an MOI of 50 for 6 h apically. No translocation was observed except the invasive reference strain *S.* Typhimurium NTB6 (Table 3.1).

Table 3.1 Translocation in airway epithelial monolayer

Unit: CFU	Time	MRSA252	MSSA NCTC 6571	*B. multivorans* 13243	*E. coli* HB101	*S.* Typhimurium NTB6
Inoculum	0 h	5.8×10^6	4.8×10^6	5.3×10^6	5.4×10^6	5.6×10^6
Basolateral chamber	2 h	0	0	0	0	1.5×10^4
	4 h	0	0	0	0	3.9×10^6
	6 h	0	0	0	0	3.8×10^7
Apical chamber	6 h	3.3×10^7	2.9×10^7	3.6×10^8	6.0×10^7	N/A

Biofilm formation is a major challenge of bacterial eradication, particularly in chronic lung infections. We showed differential biofilm development with a selection of Bcc and *S. aureus* strains. As expected, CF-associated isolates developed more robust biofilms compared to the environmental isolate J2552. *B. cenocepacia* J2315 belongs to the epidemic lineage ET12, which makes up the majority of *B. cenocepacia* isolates from CF patients and accounts for a dramatically shorter survival expectancy (Jones et al., 2004). Isolates of *B. multivorans* and *B. cenocepacia* have been shown to form stronger biofilm and are more invasive of 16HBE cells than *B. cepacia*, *B. stabilis* and *B. vietnamiensis* (Caraher et al., 2007). A study (Cunha et al., 2004) described the isolation of 15 Bcc strains from 21 CF patients and the testing the abilities of biofilm formation *in vitro*. Although the results suggested that the virulence of lung infections caused by Bcc is not correlated with biofilm formation, three patients who died during 7-year surveillance period were hosting biofilm-forming strains. Our results show that *S. aureus* strains have different abilities of biofilm development, and it is uncoupled with their abilities of IL-8 induction, which suggests the proinflammatory response of airway epithelial cells caused by *S. aureus* depends on virulence determinants other than biofilm formation.

The recognition of microorganisms is the first step of host defence. The Gram-negative pathogen *B. cenocepacia* J2315 has prototypical agonists for TLR2, 4 and 5, which are lipopeptide, LPS and flagellin, respectively (West et al., 2009); whereas Gram-positive *S. aureus* species are mainly detected by TLR2. TLR2/1 and TLR2/6 heterodimers recognise a wide range of *S. aureus* cell wall components, such as teichoic acid, peptidoglycan and lipoteichoic acid (Fournier and Philpott, 2005). The stimulation of Toll-like receptors by conserved microbial surface ligands initiates sequential events, including the activation of the NF-κB pathway, proinflammatory cytokine and chemokine secretion, and activation and recruitment of professional immune cells. We showed that *B. cenocepacia* J2315 elicited a robust IL-8 production, whereas heterogeneity was seen in *S. aureus* isolates-

induced IL-8 production. This is consistent with the finding of differential abilities of nasal *S. aureus* isolates to induce proinflammatory cytokine TNFα production by PBMCs (Peres et al., 2015). Mayer et al. (2007) demonstrate that human bronchial epithelial cell line BEAS-2B cells had dose-dependent IL-8 production in response to Gram-negative pathogens whereas they were hypo-responsive to Gram-positive bacteria such as *S. aureus*. TLR2 and its co-receptor CD36 co-transfection restored IL-8 responses to TLR2 agonists. Therefore, the expression of TLR2 and CD36 are limiting factors of the pro-inflammatory response to *S. aureus*. However, in our system, a few MSSA strains (SH1000 and RN4220) are strong inducers of IL-8 production, which suggested they might express different surface ligands.

Inhibition of PI3K activity with pharmacological inhibitors did not change IL-8 production in *S. aureus*- or Bcc-stimulated 16HBE cells, with the exception of *B. cenocepacia* J2315, suggesting only J2315 is capable of inducing a robust proinflammatory response of 16HBE cells that is PI3K-dependent. One possible explanation is the scale of IL-8 response induced by J2315 is much higher than the other strains, and therefore the supressing effect of PI3K inhibitors is inclined to be larger. The concentrations of LY294002 (30 μM) and wortmannin (100 nM) used here are typical concentrations for inhibiting PI3K activity (Davies et al., 2000). The PI3K regulation of the proinflammatory response to *B. cenocepacia* in macrophages has been reported previously (Cremer et al., 2011). *B. cenocepacia* stimulates NF-κB activity by activating PI3K/Akt signalling, followed by inactivating the downstream NF-κB repressor GSK3β. However, PI3K/Akt plays different roles in the immune response depending on the cell type and stimulus. In neutrophils, PI3K promotes migration, phagocytosis, respiratory burst and survival (Martin et al., 2015; Luo and Mondal, 2015), whereas in monocytes (Luyendyk et al., 2008), macrophages (Androulidaki et al., 2009) and dendritic cells (Fukao et al., 2002), PI3K activity negatively regulates the pro-inflammatory response to maintain homeostasis. Indeed, defining the role of PI3K in inflammation as a positive or negative regulator is oversimplified, as different PI3K and Akt isoforms finely tune the inflammatory responses in distinct ways as complex as the inflammatory response itself. For example, while Akt1 promotes M2 macrophage polarisation, Akt2 contributes to M1 phenotype polarisation (Androulidaki et al., 2009; Arranz et al., 2012; Vergadi et al., 2014). $Akt1^{-/-}$ macrophages are hypo-responsive to LPS, exhibit higher levels of iNOS and enhanced bacterial clearance (Arranz et al., 2012), whereas $Akt2^{-/-}$ macrophages are hyper-responsive to LPS, suppress TLR4 signalling through the anti-inflammatory microRNA miR-146a (Vergadi et al., 2014). Furthermore, the p110δ isoform of PI3K promotes neutrophil flux and inflammation in macrophages (Konrad et al., 2008). PI3K p110δ plays an opposing

role in bone marrow-derived dendritic cells by inducing internalisation of TLR4 and degradation of the adaptor molecule TIRAP, which switches from proinflammatory phase to anti-inflammatory phase, and thereby, enhances anti-inflammatory cytokines (IL-10 and IFN-β) production (Aksoy et al., 2012). Furthermore, PI3K p110δ is found to play a dominant role in IL-10 production in response to *S. aureus* through the PI3K/Akt/mTOR pathway (Peres et al., 2015). In this project, we intended to investigate the anti-inflammatory response to *S. aureus* and Bcc by measuring IL-10 production. However, 16HBE cells do not express IL-10 at mRNA or protein level (Massengale et al., 1999), and we could not detect any IL-10 production using ELISA (data not shown).

3.7 Conclusions

The results presented in this chapter demonstrate the host response of airway epithelial cells stimulated by *S. aureus* and *Burkholderia cepacia* complex isolates. Barrier function of polarised airway epithelial cells was disrupted by MRSA252 and *B. multivorans* 13243. *S. aureus* isolates had differential abilities of inducing IL-8 production, which did not correlate with biofilm development. *B. cenocepacia* J2315 induced a strong IL-8 production, in part through PI3K signalling. Therefore, the role of PI3K/Akt in infection process needs further investigation.

Chapter 4 Investigating the Role of PI3K/Akt, MAPK and NF-κB Signalling in Bacterial Infection of Human Airway Epithelial Cells

4.1 Introduction

Bacterial adherence to the airway lumen is an essential step of infection, followed by internalisation depending on the lifestyle of the pathogen (Silva, 2012). Multiple bacterial surface structures are involved in adhesion and invasion, such as cell wall-anchored proteins (Foster et al., 2014), the cable pili (Essex-Lopresti et al., 2005), and flagella (Bucior et al., 2012). These adhesins interact with host receptors and activate host kinase pathways in order to trigger actin rearrangement, which enables invasion and uptake of bacteria by endocytosis (Colonne et al., 2016). By doing so, intracellular bacteria are temporally protected from antimicrobial substances (such as nitric oxide, defensins, and lysozymes) in the extracellular milieu, as well as evade recognition and phagocytosis by immune cells (Eisele and Anderson, 2011). Pathogens exploit the intracellular niche as a reservoir for proliferation, and subsequent dissemination which also requires the manipulation of actin rearrangement (Colonne et al., 2016). Hence, subverting the interaction between pathogen and cytoskeleton may provide a novel antimicrobial treatment.

The microbial detectors (PRRs) are widely expressed in phagocytes and non-professional phagocytes such as epithelial cells and fibroblasts (Takeuchi and Akira, 2010). As a family of well characterised PRRs, TLRs are involved in host response to bacteria, viruses, and parasites. Activation of TLR leads to sequence of events: activation of downstream signalling cascades (including the MAPK and NF-κB pathways) (Takeuchi and Akira, 2010), nuclear translocation of transcription factors (such as NF-κB, AP-1, IRFs) (Troutman et al., 2014), and production of cytokines/chemokines and antimicrobial substances (Medzhitov, 2001; Selsted and Ouellette, 2005). In addition to the innate immune response, TLRs induce antigen uptake and presentation. Thus, TLR provides double protection to the host by (1) removing invading pathogens by directly phagocytosis and (2) triggering the adaptive immune response if the innate immune response is not sufficient to eradicate pathogens. However, bacterial pathogens have evolved several tactics to circumvent immune clearance by manipulating TLR signalling.

PI3K/Akt finely tunes TLR signalling both positively and negatively. TLR2-mediated NF-κB activation requires PI3K activity, as blocking PI3K using pharmacological inhibitors hinders phosphorylation of p65 NF-κB (Strassheim et al., 2004). While PI3K/Akt is involved in the pro-inflammatory response, this signalling cascade prevents excessive inflammation by promoting the anti-inflammatory response (Fukao and Koyasu, 2003). For example, the transcription factor FoxO1 enhances TLR4-mediated signalling, which involves activation of Akt (Fan et al., 2010b). However, activated Akt negatively regulates

FoxO1, thereby putting the brake on TLR4 signalling. Although the role of PI3K in inflammation remains controversial, it is undeniable that owing to its role in cellular proliferation and survival, PI3K is beneficial to the host during an immune response (Weichhart and Säemann, 2008).

In addition, PI3K activity is critical for cytoskeleton rearrangement and bacterial invasion (Kierbel et al., 2005; Oviedo-Boyso et al., 2011). Therefore, inhibiting PI3K activity may be a potential target to reinforce host barrier. However, although many studies have been done with myeloid cells, the role of PI3K/Akt in airway epithelial cells in response to bacterial pathogens remains unclear.

The aims of this chapter were to investigate modulation of PI3K, MAPK and NF-κB signalling by *S. aureus* and Bcc in bronchial epithelial cells, and particularly the role of PI3K in bacterial invasion.

4.2 PI3K activity regulates *S. aureus* invasion of airway epithelial cells

To assess *S. aureus* and Bcc adhesion, human airway epithelial 16HBE cells were infected by MRSA252, MSSA NCTC 6571, or *B. multivorans* 13243 at an MOI of 50 for 2 hours. *E. coli* HB101 and *S.* Typhimurium NTB6 were used as the negative and positive control, respectively. Adhered bacteria were counted by determining the viable count of the epithelial cell lysates and presented as a percentage of the inoculum (Figure 4.1A). The resulted showed that cellular adherence by MRSA252 was 1-fold higher than MSSA NCTC 6571, whereas only minimal amount of *B. multivorans* 13243 (4.6% ± 1.4%) attached to 16HBE cells. To assess *S. aureus* and Bcc internalisation, 16HBE cells were exposed to MRSA252, MSSA NCTC 6571, or *B. multivorans* 13243 (MOI 50) for 2 hours, followed by 1- or 2-hour antimicrobial treatment to eliminate extracellular bacteria. The number of internalised bacteria was determined using viable count and shown as a percentage of adherence (Figure 4.1B). Although less amount of MSSA NCTC 6571 and *B. multivorans* 13243 adhered on epithelial cells, their percent of internalisation was higher than MRSA252.

To investigate the role of PI3K/Akt in bacterial adherence and invasion, two structurally distinct pharmacological inhibitors of PI3K, LY294002 (Vlahos et al., 1994) and wortmannin (Arcaro and Wymann, 1993) were used. In Figure 4.2A and B, 16HBE cells were treated with 10 μM LY294002 30 minutes prior to bacterial infections. LY294002 upregulated marginally the adherence of MRSA252 and *S.* Typhimurium NTB6, whereas *B. multivorans* 13243 attachment was reduced by 60%. Only minimal numbers of MRSA252 and MSSA NCTC 6571 entered into epithelial cells, and this was decreased

with LY294002. The invasion assay was also performed with a higher concentration of LY294002 (30 μM) and wortmannin (100 nM) (Figure 4.2C and D). MRSA252 and MSSA NCTC 6571 maintained the same level of adherence, whereas LY294002 caused a modest reduction, and wortmannin caused a modest increase of adherence of *B. multivorans* 13243. LY294002 reduced the invasion of MRSA252, MSSA NCTC 6571 and *B. multivorans* 13243 by more than 50%. In contrast, wortmannin only caused a decrease for *B. multivorans* 13243. Further invasion assays were performed with Bcc isolates (Figure 4.2E and F). In comparison with the CF-related pathogens *B. multivorans* 13243 and *B. cenocepacia* J2315, the environmental isolate *B. anthina* J2552 did not adhere to epithelial cells. Although the pretreatment of PI3K inhibitors was extended to 1 hour, no effect was observed on adherence. Internalisation of *B. anthina* J2552 was blocked by LY294002. Altogether, in the process of invasion, PI3K is not involved in adherence but is involved in internalisation, particularly for *S. aureus*.

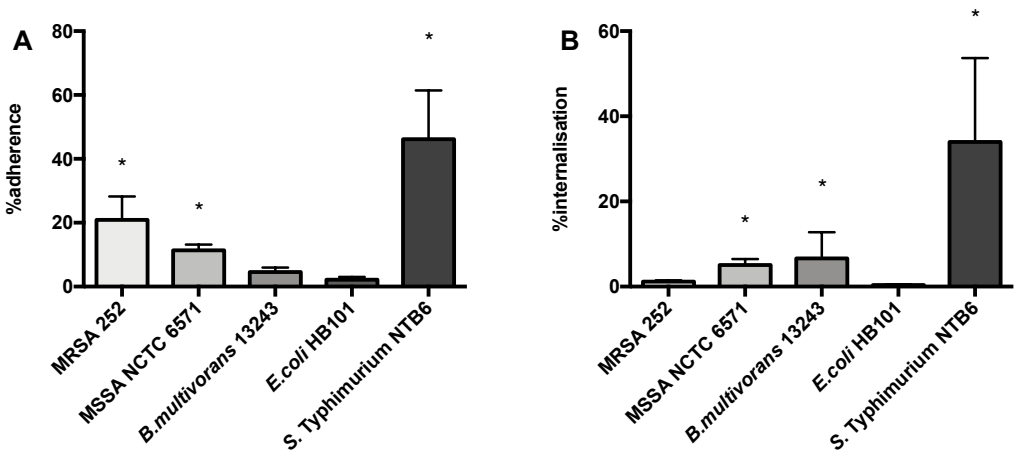

Figure 4.1 Pilot study of adherence to and invasion of airway epithelial 16HBE cells.

(A) Adherence of MRSA252, MSSA NCTC 6571, or *B. multivorans* 13243 to 16HBE cells at 2 hours post infection (MOI 50). *E. coli* HB101 and *S.* Typhimurium NTB6 were used as the negative control and the positive control, respectively. **(B)** Invasion of 16HBE cells by MRSA252, MSSA NCTC 6571, or *B. multivorans* 13243 was quantified after 1 or 2 hours antibiotic treatment. Data are presented as mean ± SEM from three independent experiments performed in duplicate. *, $p<0.05$ compared to *E. coli* HB101, using two-way ANOVA followed by Dunnett's multiple comparisons test.

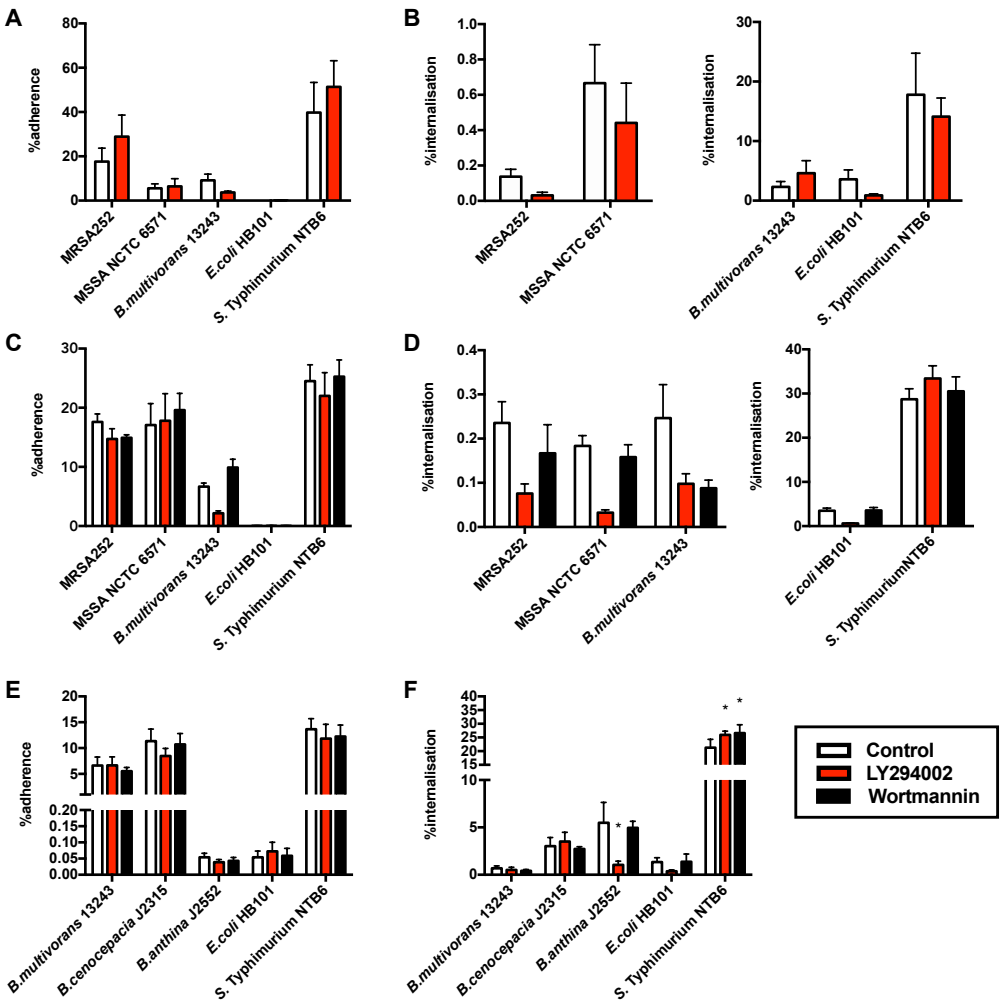

Figure 4.2 Inhibition of bacterial adherence and internalisation by pharmacological inhibitors of PI3K.

(A and B) After 30 minutes treatment with LY294002 (10 μM), 16HBE cells were infected with MRSA252, MSSA NCTC 6571, *B. multivorans* 13243, *E. coli* HB101 or *S.* Typhimurium NTB6 (50 MOI) for 2 hours with the presence of LY294002. 16HBE cells were washed 3 times with DPBS to remove nonadherent bacteria and subsequently lysed in 1% saponin (100 μL) to quantify adhered bacteria using viable count. The percentage of adhered bacteria was normalised against the inoculum. Alternatively, extracellular adherent bacteria were killed with antibiotics for another hour (2 hours for Bcc strains) to measure the amount of internalised bacteria. The percentage of internalised bacteria was calculated against the number of adhered bacteria. Adherence **(A)** and invasion **(B)** are shown as mean ± SEM from four independent experiments, each performed in duplicate. Data were analysed using two-way ANOVA following Bonferroni's multiple comparisons test. *, $p<0.05$.

(C and D) After 30 minutes treatment with LY294002 (30 μM) or wortmannin (100 nM), 16HBE cells were challenged with MRSA252, MSSA NCTC 6571, *B. multivorans* 13243, *E. coli* HB101 or *S.* Typhimurium NTB6 (50 MOI) with the presence of inhibitors. Adherence **(C)** and invasion **(D)** were assessed as previous and are shown as mean ± SEM from three independent experiments in duplicate. Data were analysed using repeated measures two-way ANOVA following Dunnett's multiple comparisons test.

(E and F) After 1 hour treatment with LY294002 (30 μM) or wortmannin (100 nM), 16HBE cells were infected with *B. multivorans* 13243, *B. cenocepacia* J2315, *B. anthina* J2552, *E. coli* HB101 or *S.* Typhimurium NTB6 (50 MOI) with the presence of inhibitors. Adherence **(E)** and invasion **(F)** were assessed as previous and are shown as mean ± SEM from three independent experiments, each performed in duplicate. Data are analysed using repeated measures two-way ANOVA following Dunnett's multiple comparisons test.

4.3 Modulation of PI3K/Akt signalling by *S. aureus* isolates

Akt is the major downstream target of PI3K and activated at Ser473 and Thr308 through a dual phosphorylation mechanism. In order to study the role of PI3K, airway epithelial 16HBE cells were infected with planktonic (Figure 4.3) or biofilm (Figure 4.4) MRSA252 or MSSA NCTC 6571 cultures, and phospho-Akt (Ser473) and phospho-Akt (Thr308) were analysed by immunoblotting. Although planktonic *S. aureus* cultures were used at a high dose (10^8 CFU/mL), they failed to activate Akt from 5 to 30 minutes. With their biofilm counterparts, phospho-Akt (Ser473) peaked at 15 minutes but no significant difference was observed (Figure 4.4A and C).

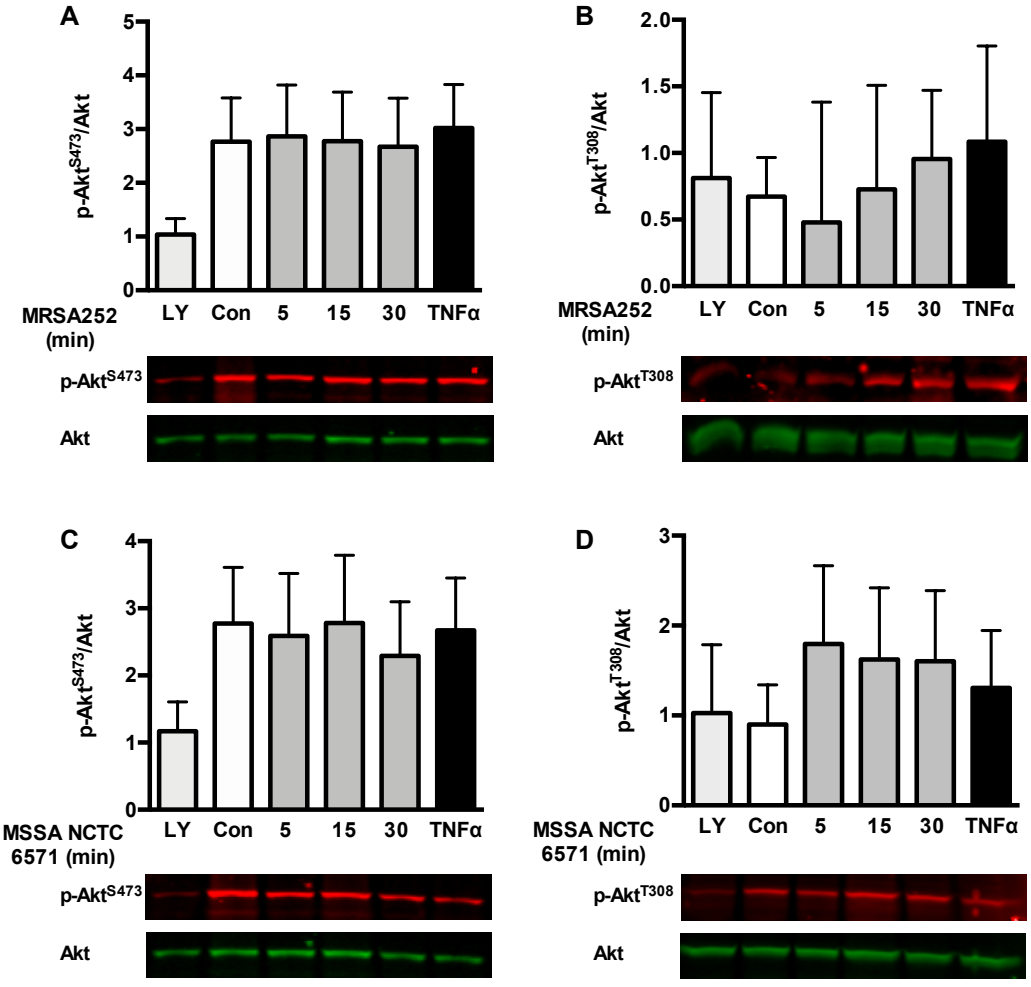

Figure 4.3 Planktonic MRSA252 or MSSA NCTC 6571 do not affect PI3K activity.

Human airway epithelial 16HBE cells were starved overnight. LY294002 (10 μM) overnight was used as negative control and TNFα (100 ng/mL) for 15 minutes was used as positive control. Cells were infected with MRSA252 or MSSA NCTC 6571 culture (10^8 CFU/mL) for the time indicated. Cell lysates were analysed by immunoblotting to quantify phospho-Akt (Ser473 and Thr 308). The fluorescence signals were normalised against total Akt and are presented as mean ± SEM from three independent experiments.

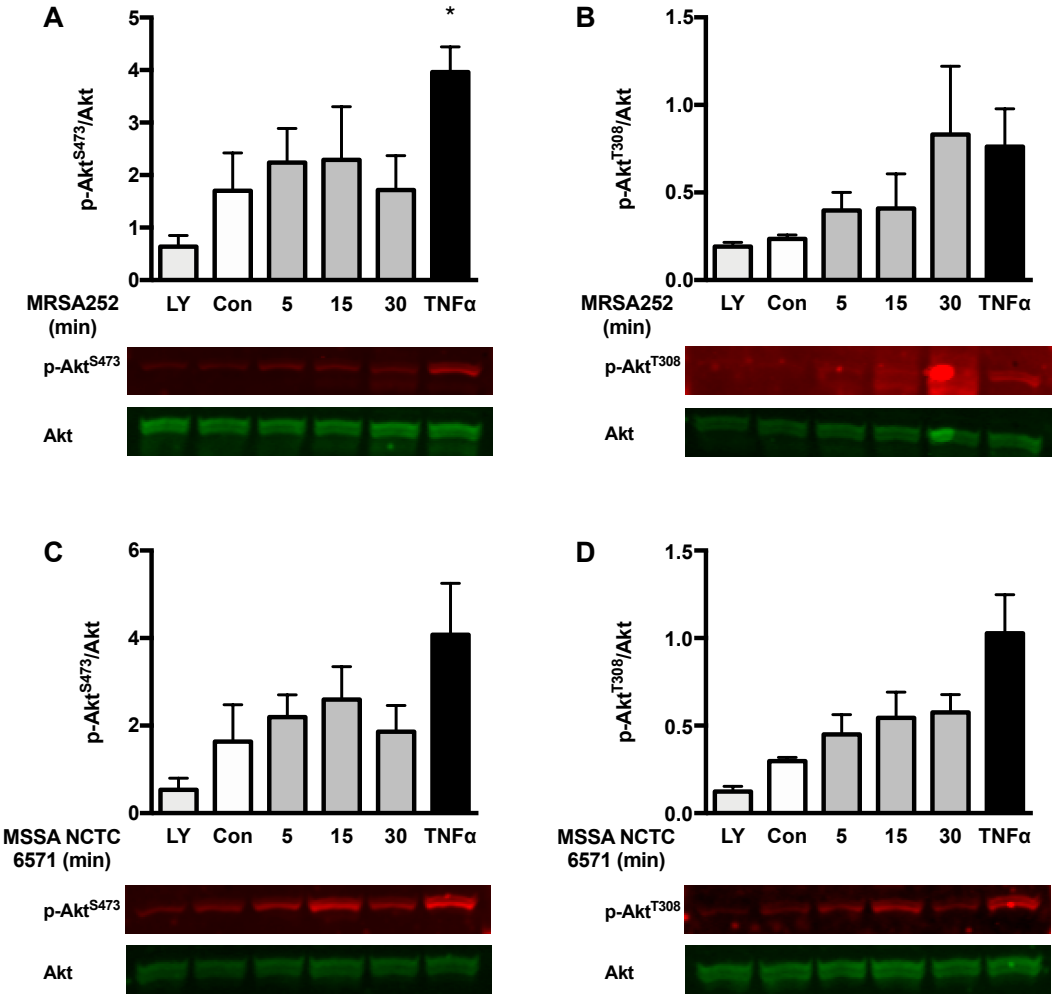

Figure 4.4 Biofilm MRSA252 or MSSA NCTC 6571 do not affect PI3K activity.

Human airway epithelial 16HBE cells were starved overnight. LY294002 (10 μM) overnight was used as the negative control and TNFα (100 ng/mL) for 15 minutes was used as the positive control. Cells were infected with MRSA252 or MSSA NCTC 6571 culture (10^8 CFU/mL) for the time indicated. Cell lysates were analysed by immunoblotting to quantify phospho-Akt (Ser473 and Thr308). The signals were normalised against total Akt. Data are presented as mean ± SEM from three independent experiments. *, $p<0.05$ compared with the control using repeated measures one-way ANOVA followed by Dunnett's multiple comparisons test.

4.4 *B. cenocepacia* J2315 and *S. aureus* supernatants modulating PI3K/Akt, MAPK and NF-κB signalling in human airway epithelial cells

To investigate the effect of *B. cenocepacia* on MAPK signalling, 16HBE cells were challenged with the robust IL-8 inducer J2315 (Figure 3.3), at various MOIs (Figure 4.). With an MOI of 5, one-fold upregulation of phosphorylation of p38 was observed at 30 and 60 minutes post infection. In contrast, phosphorylation of Erk was not changed by any treatment. With 50 MOI, the level of phosphorylation of p38 and Erk did not change. J2315 failed to activate NF-κB signalling, as no increase in phosphorylation of IκBα was observed. Furthermore, higher MOI (100) of J2315 did not activate phosphorylation of IκBα or p65.

Since it has been reported that *S. aureus* supernatants suppress TLR2 agonist Pam_3CSK4-activated NF-κB pathway in human bronchial epithelial cells, to replicate this in our system, 16HBE cells were stimulated with different concentrations of MSSA NCTC 6571 supernatant (Chekabab et al., 2015). When the cells were treated with lower dilution of MSSA NCTC 6571 supernatant (10% v/v, Figure 4.A and B), only a minimal increase in phosphorylation of p38 was observed at 60 minutes post stimulation, whereas phosphorylation of Erk was significantly inhibited at 15 minutes. In contrast, 25% (v/v) MSSA NCTC 6571 supernatant downregulated phosphorylation of p38 at 5 and 30 minutes, and recovered back to baseline at 240 minutes (Figure 4.C). The bacterial culture medium trypticase soy broth (TSB, 10% v/v, 2 hours stimulation) has previously been shown to significantly downregulate Erk signalling in airway epithelial S9 cells but not 16HBE cells (Below et al., 2009). In Figure 4.C and D, 4 hours treatment with 25% (v/v) LB did not change phosphorylation of p38 but increased phosphorylation of Erk by 50%. This suggests the upregulation of phospho-Erk may result from LB but not *S. aureus* supernatant.

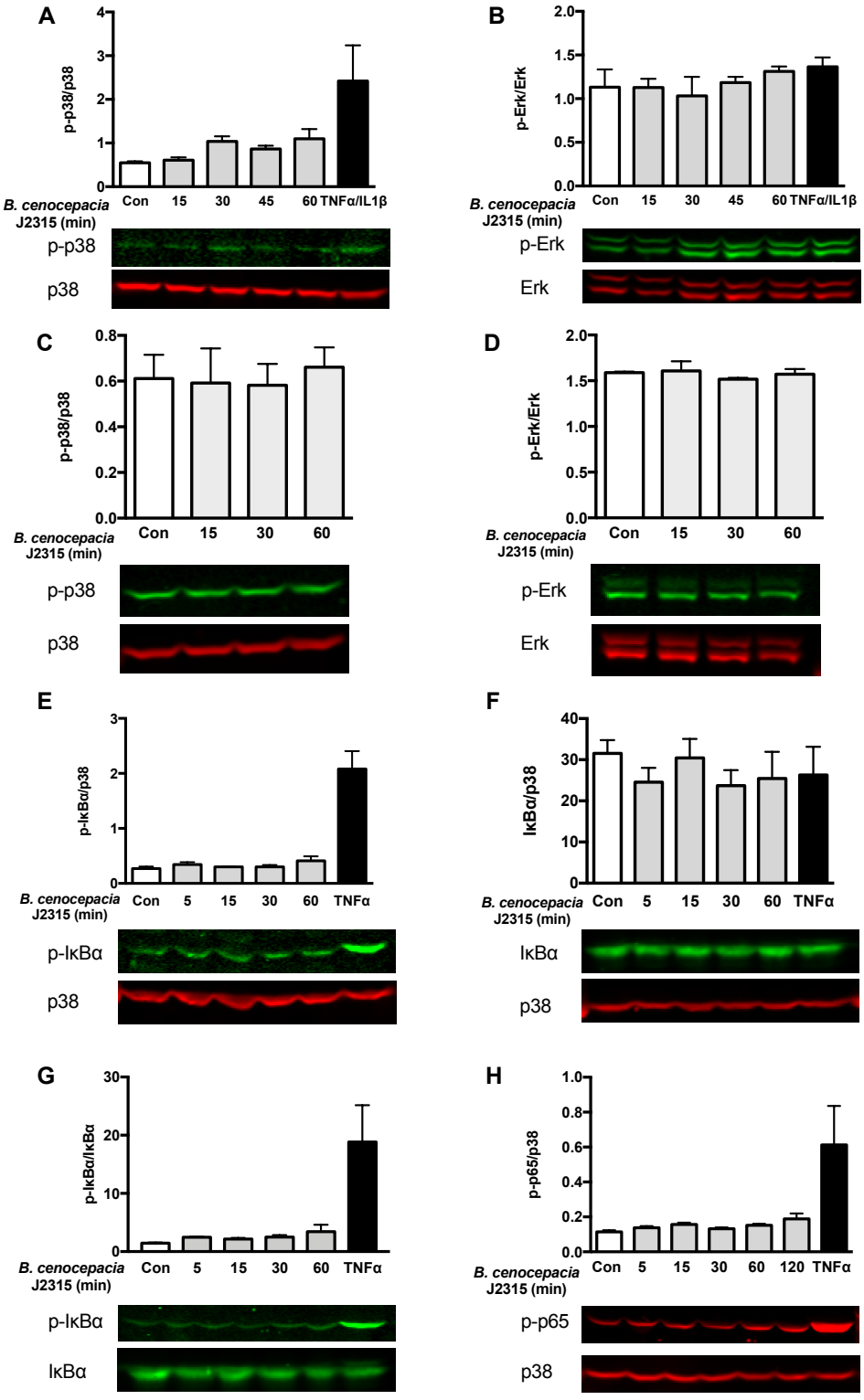

Figure 4.5 *B. cenocepacia* J2315 has no effect on MAPK or NF-κB signalling.

Airway epithelial 16HBE cells were stimulated with *B. cenocepacia* J2315 at various MOIs (**A and B**, MOI=5; **C-F**, MOI=50; **G and H**, MOI=100) for the indicated time. **(A and B)** TNFα (20 ng/mL) and IL1β (20 ng/mL) for 20 minutes were used as the positive control. **(E-H)** TNFα (20 ng/mL) for 5 minutes was the positive control. Phosphorylation of p38, Erk, IκBα, and p65 were detected by western blotting and the fluorescence signals were normalised against p38. Data are shown as mean ± SEM from three independent experiments and analysed using repeated measures one-way ANOVA following Dunnett's multiple comparisons test.

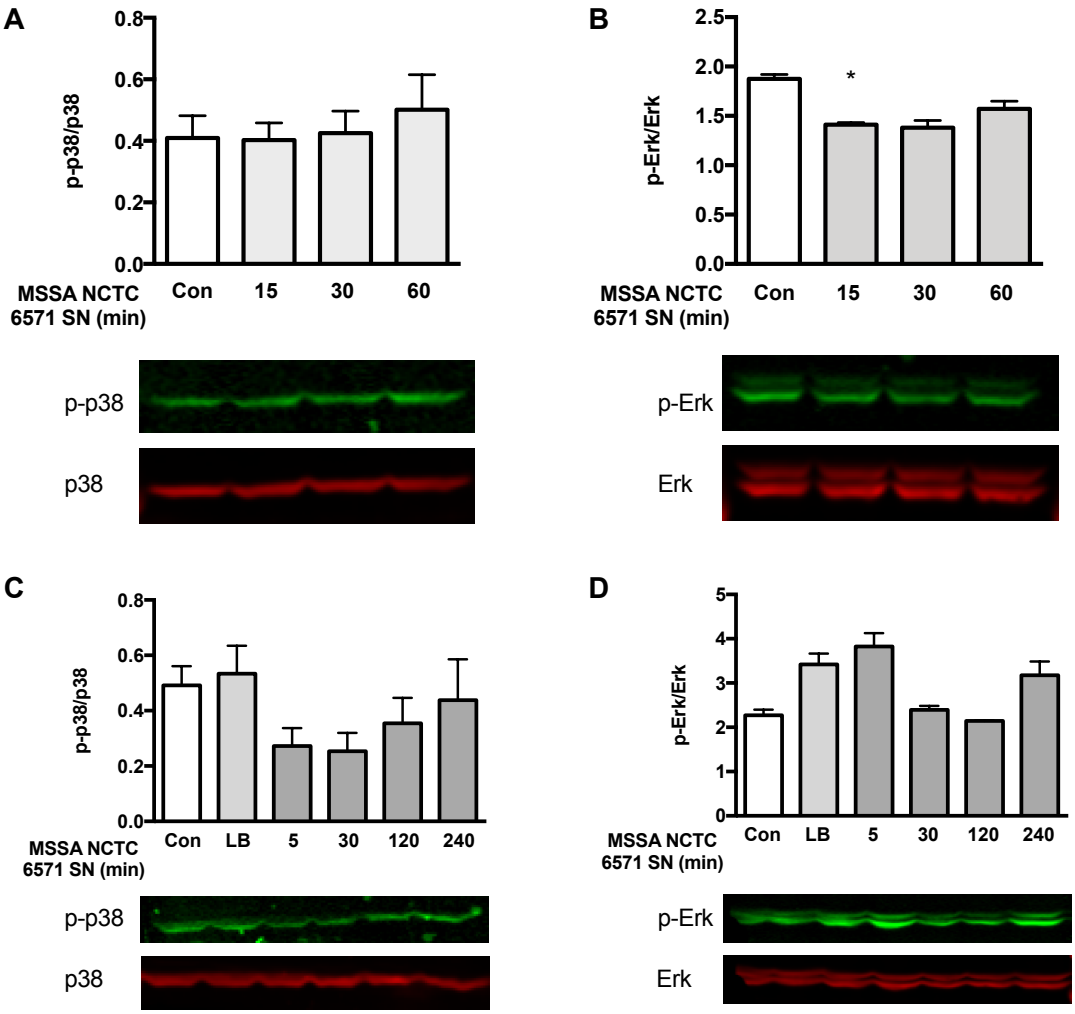

Figure 4.6 Analysis of Erk and p38 by MSSA NCTC 6571 supernatant.

16HBE cells were incubated with 10% (v/v) **(A and B)** or 25% (v/v) **(C and D)** MSSA NCTC 6571 conditioned medium for the indicated time. In **C and D**, cells were treated with 25% v/v LB medium for 4 hours as a control for LB. Western blots on cell lysates were done to measure phospho-p38, phospho-Erk. Membranes were reprobed with loading control total p38 and Erk antibodies. Data are shown as mean ± SEM from three independent experiments. *, $p<0.05$ compared with the control using repeated measures one-way ANOVA following Dunnett's multiple comparisons test.

To optimise the dose of stimuli, 16HBE cells were treated with increasing dose of *B. cenocepacia* J2315 or MSSA NCTC 6571 supernatant for 1 hour (Figure 4.7). A dose-dependent increasing trend was observed with J2315. In contrast, *S. aureus* supernatant failed to regulate Erk signalling.

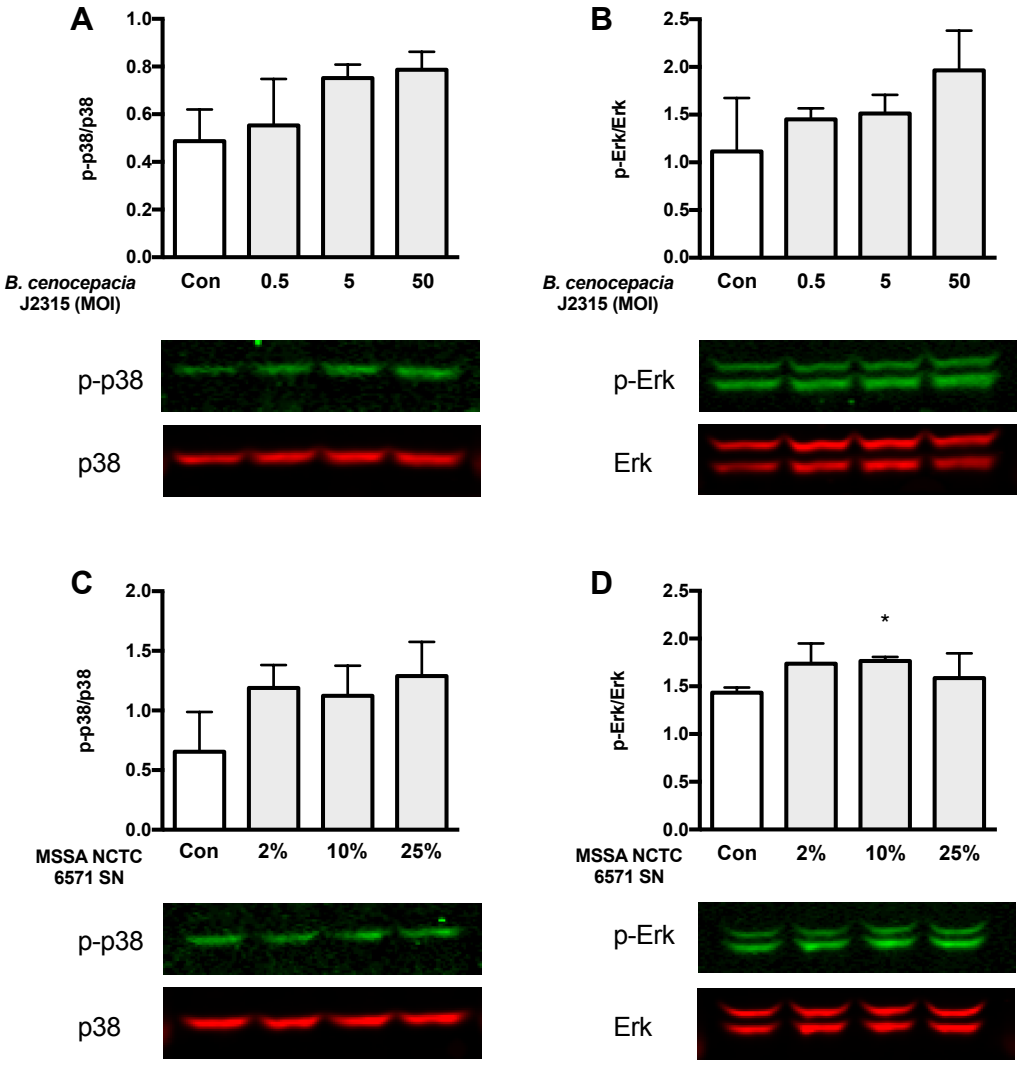

Figure 4.7 MAPK response to *B. cenocepacia* J2315 live bacteria or MSSA NCTC 6571 supernatant.

Phosphorylation of p38 and Erk of 16HBE cells after stimulated with *B. cenocepacia* J2315 **(A and B)** or MSSA NCTC 6571 supernatant (v/v, **C and D**) for 1 hour. Phospho-p38 and phospho-Erk of cell lysates were measured by western blot. Membranes were reprobed with loading control total p38 and Erk antibodies. Data are shown as mean ± SEM from three independent experiments and analysed using repeated measures one-way ANOVA following Dunnett's multiple comparisons test. *, $p<0.05$.

As surface structures (such as lipopeptides, flagellin and LPS) of *B. cenocepacia* play important roles in host-pathogen interactions, the potentially damaging effect of centrifugation in the bacteria preparation process should be considered (Peterson et al., 2011). In Figure 4., 16HBE cells were infected with unwashed J2315 at an MOI of 5 for up to 180 minutes and phosphorylation of Akt was observed with maxima at 120 minutes. The Akt downstream target GSK-3β was phosphorylated marginally compared to the control. For the MAPK family, phosphorylation of p38 showed a 1-fold increase at 120 minutes, whereas phosphorylation of Erk peaked at 5 minutes, followed by a decrease and then a secondary rise at 120 minutes. For NF-κB signalling, J2315 upregulated phosphorylation of p65, particularly after 120 minutes. Except for NF-κB, TNFα failed to activate PI3K/Akt and MAPK signalling as a positive control. Due to differential signalling response to unwashed J2315 culture comparing with washed culture (Figure 4.), a further experiment was performed to directly compare the difference. As shown in Figure 4., washed and unwashed J2315 cultures were used to stimulate 16HBE cells for 5 and 120 minutes. Although no significant difference was shown in phosphorylation of Akt and GSK-3β, a higher level of phosphorylation of Akt was observed with unwashed culture at 120 minutes. Both washed and unwashed cultures had 2-fold higher phosphorylation of p38 at 120 minutes compared with the control (Figure 4.C). In contrast, with washed culture, phosphorylation of Erk showed a 1-fold increase comparing 120 to 5 minutes, whereas with unwashed culture a 1-fold increase in phosphorylation of Erk exhibited both at 5 and 120 minutes, which is consistent with Figure 4.D. Similar to the previous results, washed and unwashed culture induced robust phosphorylation of p65 at 120 but not 5 minutes.

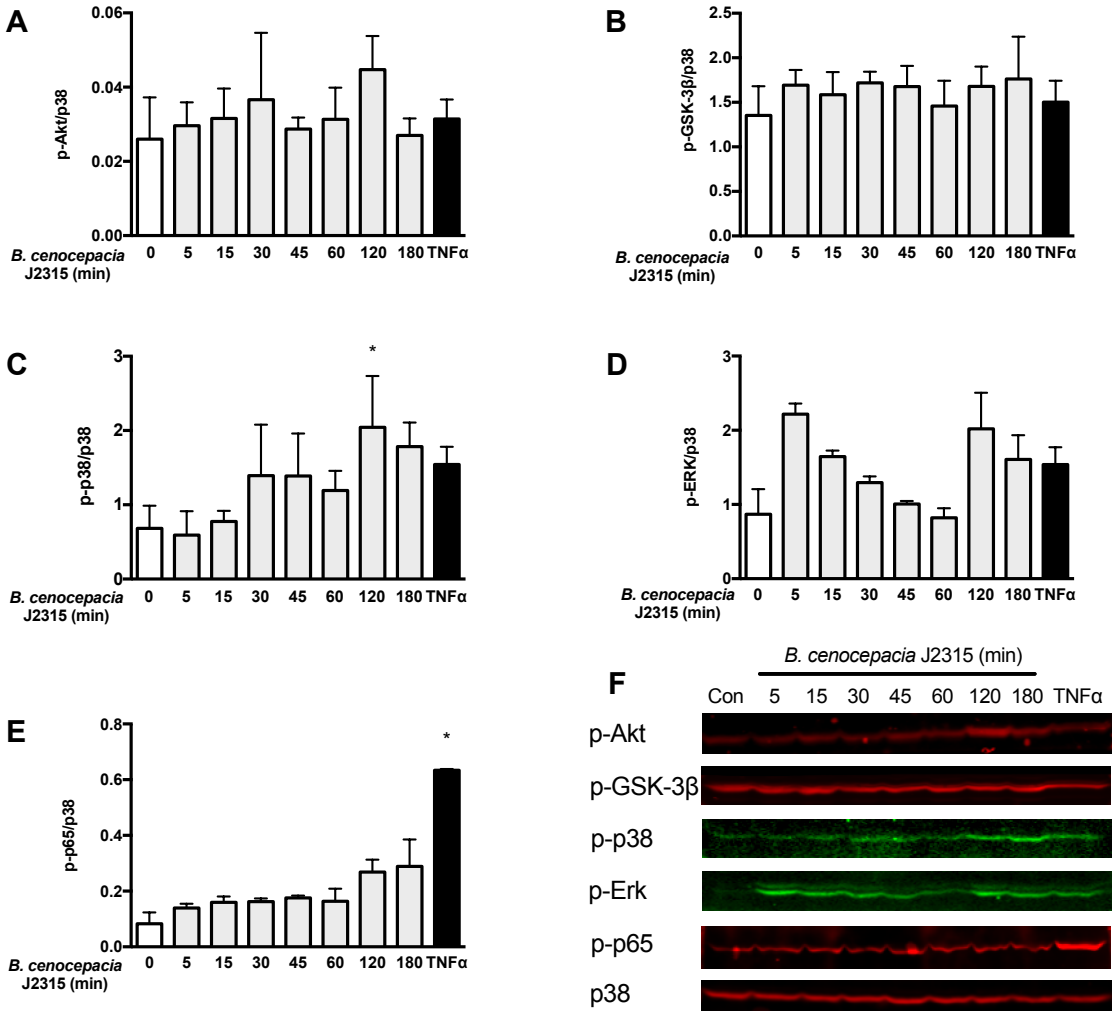

Figure 4.8 Host signalling modulation by unwashed *B. cenocepacia* J2315 culture.

16HBE cells were infected by *B. cenocepacia* J2315 unwashed culture at an MOI of 5 for the indicated time. TNFα (20 ng/mL) for 5 minutes was used as the positive control. Phosphorylation of Akt **(A)**, GSK-3β **(B)**, p38 **(C)**, Erk **(D)**, p65 **(E)** of cell lysates were measured by western blot and normalised against total p38. **(F)** Western blot analysis for levels of phosphorylated Akt, phosphorylated GSK-3β, phosphorylated p38, phosphorylated Erk, phosphorylated p65, and p38 in cell lysate following *B. cenocepacia* J2315 stimulation. Protein bands representative of three independent experiments. Data are shown as mean ± SEM. *, $p<0.05$ compared with the control using repeated measures one-way ANOVA following Dunnett's multiple comparisons test.

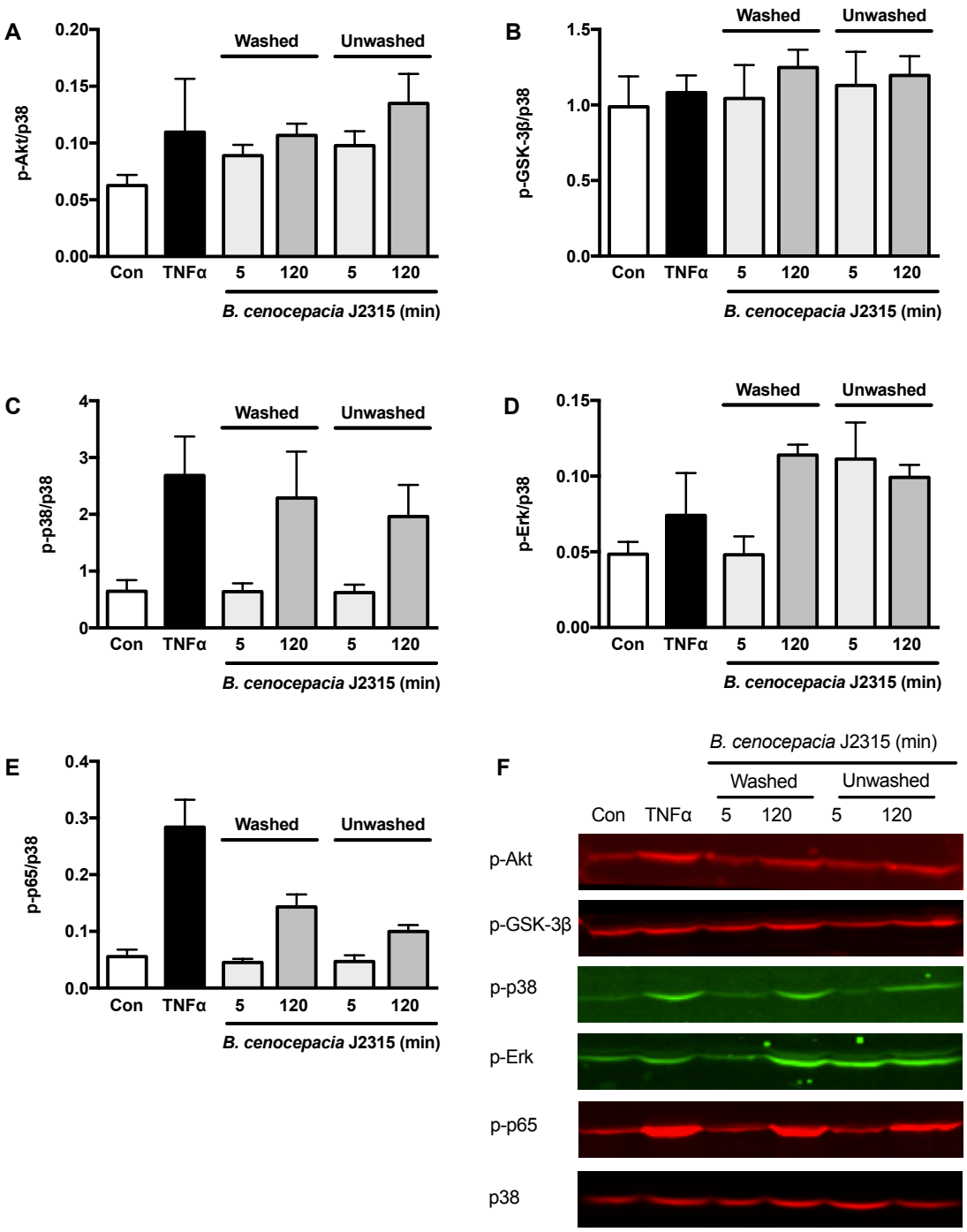

Figure 4.9 Comparison of washed and unwashed *B. cenocepacia* J2315 culture on stimulating 16HBE signalling.

Overnight culture of *B. cenocepacia* J2315 were cultured into exponential phase. The culture was divided into two portions. Washed culture was centrifuged to collect bacteria and washed by DPBS once. Washed and unwashed culture were adjusted to the same optical density. 16HBE cells were challenged by *B. cenocepacia* J2315 at an MOI of 5 for 5 or 120 minutes. TNFα (20 ng/mL) for 5 minutes was used as the positive control. Phosphorylation of Akt **(A)**, GSK-3β **(B)**, p38 **(C)**, Erk **(D)**, p65 **(E)** of cell lysates were measured by western blot and normalised against total p38. **(F)** Western blot analysis for levels of phosphorylated Akt, phosphorylated GSK-3β, phosphorylated p38, phosphorylated Erk, phosphorylated p65, and p38 in cell lysate following *B. cenocepacia* J2315 stimulation. Protein bands representative of three independent experiments. Data are shown as mean ± SEM. *, $p<0.05$ compared with the control using repeated measures one-way ANOVA following Tukey's multiple comparisons test.

4.5 Discussion

In this chapter, we investigated cellular invasion and signalling cascades mediated by *S. aureus* and Bcc isolates in human bronchial epithelial 16HBE cells.

S. aureus is identified as an extracellular pathogen associated with skin and soft tissue infections in the traditional classification, whereas Bcc are dual intracellular/extracellular style pathogens (Wiersinga et al., 2006). However, there is growing evidence of an intracellular phase of *S. aureus* (Garzoni and Kelley, 2009). Here we assessed invasion by *S. aureus* and *B. multivorans,* as well as two control strains, in 16HBE cells. At 2 hours post infection, adherence of MRSA252, MSSA NCTC 6571 and *B. multivorans* 13243 showed 45%, 25% and 10% of *S.* Typhimurium NTB6, respectively, whereas internalisation was 3%, 15% and 20%. This suggests their infective lifestyles in the host are consistent with the previous study (Silva, 2012). Phosphoinositide in the cell is essential for membrane dynamics, actin polymerisation and vesicle trafficking. Therefore, by disrupting phosphoinositide homeostasis, pathogens are able to enhance their uptake by host cells (Ham et al., 2011). Gessain et al. (2015) gave further insight into the critical role of PI3K. Despite no intrinsic PI3K activity in placental epithelial cells, a surface protein of *Listeria monocytogenes* InlB activates PI3K/Akt, which enables bacterial invasion (Gessain et al., 2015),

To elucidate the role of PI3K in airway epithelial cell membrane permissiveness, we pretreated 16HBE cells with PI3K specific inhibitors LY294002 or wortmannin, followed by an invasion assay. LY294002 inhibits PI3K activity by competing the ATP binding site of catalytic subunit p110 reversibly, whereas wortmannin irreversibly causes covalent modification of p110 kinase and leads to a conformation rearrangement (Walker et al., 2000). LY294002 (30 minutes treatment) failed to affect adherence of tested isolates, with the exception of *B. multivorans* 13243. However, the downregulation effect was not consistent with a longer pretreatment period (1 h). This part requires more replicates to validate the results. Of note, although only minimal amounts of *S. aureus* entered into epithelial cells, a consistent decrease was seen with LY294002, suggesting internalisation of *S. aureus* requires PI3K activity. This result is consistent with a study working with polarised MDCK cells and *P. aeruginos*a strain K (Kierbel et al., 2005). In addition, although pharmacological inhibition of Akt subverts *S. aureus* entry into endothelial cells as well, inhibition of Akt phosphorylation on Thr308 residue does not affect entry, suggesting *S. aureus* internalisation may be associated with phosphorylation of Akt on Ser473 (Oviedo-Boyso et al., 2011). Unexpectedly, although wortmannin is a more potent (IC50=5 nM) PI3K inhibitor compared with LY294002 (IC50=1.4 μM), in our model

wortmannin failed to cause changes similar to LY294002. We found similar results in Figure 3.4, in which wortmannin did not reduce bacteria-induced IL-8, except for *B. cenocepacia* J2315. This might be due to the instability of wortmannin in the presence of nucleophiles (Yuan et al., 2007).

PI3K/Akt, MAPK and NF-κB are known to play critical roles in response to bacterial pathogens (Krachler et al., 2011). However, pathogens have evolved to exploit these pathways in order to promote survival and proliferation inside the host. First, we investigated activation of PI3K by planktonic or biofilm culture of *S. aureus* isolates. 16HBE cells endogenously express phosphorylated-Akt at a relative high level, which might leave a very small window of unphosphorylated-Akt for *S. aureus*. This might explain why no visible trend was seen in cells stimulated with *S. aureus*. Then we assessed the ability of *B. cenocepacia* J2315 to activate PI3K. Activation of Akt leads to phosphorylation and inactivation of GSK-3β (Ser9). Phosphorylation of Akt (Ser473) peaked at 2 hours, but did not upregulate phosphorylation of GSK-3β. As a key regulator of numerous cellular functions, positive or negative dysregulation of PI3K/Akt in epithelial cells by bacteria has been reported in many studies. For example, invasion of *P. aeruginosa* to either the apical or basolateral surface of polarised airway epithelial Calu-3 cells via pili or flagellin requires PI3K/Akt signalling, and ultimately leads to entry into host cells (Bucior et al., 2012). Apart from invasion, PI3K is a bacterial target to manipulate programmed cell death, depending on the cell types and stage of infection (Knodler et al., 2005). At early stages of infection, the *Salmonella* effector SopB is proved to be anti-apoptotic by continual activation of Akt in HeLa cells, whereas in macrophages, the effector SipB induces cell death in macrophages, thereby escaping phagocytosis (Hernandez et al., 2003).

We further performed a pilot study of the time and dose response to *B. cenocepacia* J2315 and *S. aureus* cell-free supernatant on host signalling. As IL-8 expression is associated with NF-κB (Elliott et al., 2001) and MAPK signalling pathways (Bhattacharyya et al., 2011), these pathways might be involved in *B. cenocepacia* and *S. aureus* infections. The *B. cenocepacia* ET-12 lineage strain K56-2 has been reported to significantly upregulate phosphorylation of Akt, Erk and NF-κB at an MOI of 5 in 16HBE cells within 30 minutes of stimulation (Gillette et al., 2013). In parallel, *B. cenocepacia* BC7 (isolated from a CF patient who died of cepacia syndrome and is able to induce a robust IL-8 response), triggers phosphorylation of p38 and JNK in immortalised CF bronchial epithelial IB3 cells, whereas an environmental *B. cepacia* strain fails to show any activation of these pathways (Sajjan et al., 2008). This indicates that activation of MAPK signalling is related to more intense lung inflammation caused by Bcc isolates.

In contrast, in our system *B. cenocepacia* J2315 failed to active these pathways even at a high MOI. During the process of preparing the strain, in order to remove metabolites and secreted products, bacterial cultures were centrifuged twice (5000 *g* for 10 minutes), which might potentially cause damage to bacterial surface structures. Therefore, we performed same experiments using J2315 culture without centrifugation steps. Phosphorylation of Akt, p38, Erk and p65 increased at 120 minutes post-infection. Of note, J2315 without the centrifugation step caused a transient Erk phosphorylation at 5 minutes post-infection and peaked again at 120 minutes, which was reproduced in Figure 4.9. Centrifugation is likely to damage surface structures such as LPS and flagellin. D'Elia et al. observed a similar bi-phasic activation pattern of Erk and p38 in response to *B. pseudomallei in vitro* and *in vivo* (D'Elia et al., 2017). Thirty-minutes post-infection, murine alveolar macrophages exhibit transient phosphorylation of p38 and Erk1, and at 2 h post-infection exhibited sustained activation. D'Elia et al. (2017) proposed that the transient activation at the early stage is associated with host defence and during the latter stage of infection host cells might initiate cell death as indicated by continuous Erk activation. *B. pseudomallei* might modulate the dynamics of Erk activation to delay apoptosis, thereby contributing to intracellular survival. Another member of MAPK family, JNK, has shown to be involved in J2315-induced TNFα and IL-1β production in macrophages. In our experiments we were unable to detect phosphorylation of JNK. In addition, we investigated the effect of *S. aureus* supernatant on host signalling cascades, as it has been reported that *S. aureus* supernatant is able to induce different cellular response in human tracheal gland cells compared to live bacteria, particularly a huge upregulation of IL-6 and IL-8 production (Moreilhon et al., 2005). However, in our system, MSSA NCTC 6571 supernatant failed to show consistent modulation on MAPK signalling. Although same conditions (10% v/v, 60 minutes stimulation) were used in Figure 4.6A and B and Figure 4.7C and D, the downregulation on phosphorylation of Erk was not reproduced. This might be caused by repeated propagation of cell lines, or phenotype variance of bacterial culture.

4.6 Conclusion

In this chapter we assessed *S. aureus* and Bcc isolates to adhere to and enter into airway epithelial cells. Of note, *S. aureus* internalisation but not adhesion is PI3K-dependent. Hence, PI3K is a critical target for barrier permissiveness by *S. aureus*. In addition, a preliminary study on the ability of *S. aureus,* Bcc live bacteria and *S. aureus* supernatant to modulate host signalling was carried out. PI3K/Akt, MAPK and NF-κB signalling was unchanged when 16HBE cells were infected with bacterial cultures prepared with

centrifugation, whereas undamaged *B. cenocepacia* J2315 culture induced a bi-phasic activation of Erk during the early and the latter stage of infection. This may indicate that *B. cenocepacia* suppresses Erk activity to delay host apoptosis in order to promote survival and proliferation.

Chapter 5 Investigating Host Response to Polymicrobial Infections in The Airway

5.1 Introduction

Over the past decades, abundant research has focused on single-species microorganisms and single virulence factors. However, the complex nature of infectious diseases such as airway infections is often associated with multiple species including bacteria, fungi and viruses. Yet we have much less understanding regarding polymicrobial communities within the infection sites, the net impact on progression and manifestations of infectious diseases and host responses to polymicrobial infections. Hence, elucidating the mechanisms of host-pathogen interactions in the context of polymicrobial communities is required to protect against such infection diseases.

Synergism and antagonism are terms that can be applied to describe the cooperative / competitive polymicrobial interactions in disease progression. According to Murray et al. (2014), synergism is 'an interaction of two or more microbes in an infection site that results in enhanced disease compared to infections containing the individual microbe acting alone'. In contrast, the definition of antagonism is 'the presence of two or more species protects the host from disease that occurs when only one species is present' (Nguyen and Oglesby-Sherrouse, 2016). Synergistic interactions between *S. aureus* and *P. aeruginosa* have been discovered in many studies, including increased antimicrobial tolerance and enhanced virulence (Dalton et al., 2011; DeLeon et al., 2014; Fugère et al., 2014; Hoffman et al., 2006). For example, clinical isolates of *P. aeruginosa* and *B. cenocepacia* co-culture exhibit stronger biofilm formation and elicit increased pro-inflammatory responses in a murine chronic lung infection model (Bragonzi et al., 2012). Conversely, *S. aureus* has demonstrated an antagonistic effect in polymicrobial infections. In a 5-year predictive multivariate survivorship model of cystic fibrosis patients, Liou et al. (2001) discussed some known risk factors such as age, gender, and frequency of acute pulmonary exacerbations, and revealed that harbouring *S. aureus* promotes survivorship, whereas *B. cepacia* has a negative effect on survivorship. Indeed, Bcc species are notoriously associated with 'cepacia syndrome', which is characterised with rapid lung function decline and mortality, particularly *B. cenocepacia* (Boussaud et al., 2008). However, it is peculiar that *S. aureus* has protective effects and the reason remains unclear. It may be due to low-level pro-inflammatory or even anti-inflammatory property of *S. aureus*, partially antagonising excessive inflammation and tissue damage resulting from *P. aeruginosa* and Bcc infections (Chavakis et al., 2007). Consistent with this finding, a study by Ahlgren et al. (2015) compared clinical outcomes (for example, lung function and exacerbation rate) of a cohort of 84 CF adults grouped into *S. aureus* infection only, *P. aeruginosa* infection only, and neither *S. aureus* nor *P. aeruginosa*. Patients with *S.*

aureus infections had a better clinical outcome and the presence of *S. aureus* appeared a marker of less severe disease in patients compared to those who are infected with *P. aeruginosa* (Ahlgren et al., 2015).

S. aureus is known to possess a plethora of immunomodulation strategies. For example, cell wall component SpA is able to activate TNFR1, elicit IL-8 expression and an excessive inflammatory response and tissue damage (Gomez et al., 2004). *S. aureus* also manipulates host responses by blocking neutrophil recruitment (Spaan et al., 2013b), phagocytosis and completement activation (Jongerius et al., 2010b; Jusko et al., 2014), as well as disrupting the adaptive immune response (Wang et al., 2012). Hence, understanding the molecular mechanism how *S. aureus* manipulates the immune system to evade microbial killing may be useful for developing novel treatments against *S. aureus* infection, particularly MRSA-related infections.

The survillence of innate immune system TLRs recognise numerous microbial ligands and trigger protective immune responses. TLRs occur either as a homodimer such as TLR4 and TLR5, or form heterodimer such as TLR1/2 and TLR2/6. All the TLRs comprise an extracellular leucine-rich repreat domain and a cytoplasmic TIR domain (Lim and Staudt, 2013). TLR and IL-1 receptor belong to the IL-1R/TLR superfamily as they contain homologous TIR domain which is responsible for signalling transduction, and thus signalling pathways are highly conserved between TLRs and IL-1R (Cohen, 2014). IL-1R and all TLRs except TLR3 recruit MyD88 as an adaptor protein, and ultimately lead to activation of NF-κB. Upon activation of TLRs, MyD88 is recruited to the receptors via its TIR domain, and interacts with IRAK1/2/4 via its death domain. Phosphorylation of IRAKs results in the activation of TRAF6, which is an E3 ubiquitin ligase. TRAF6, together with UEV1A and UBC13, synthesises ubiquitin chain and subsequently activates the kinase complex containing TAB1/TAB2/TAK1, which contributes to the activation of MAPK family and nuclear translocation of transcription factors (such as NF-κB and AP-1) and ultimately mounts a pro-inflammatory response (Chen, 2005). Modulation of any signal molecules would affect the intensity and duration of the response and cause harmful outcomes. Therefore, it is vital to fine tune TLR-mediated innate immune response to ensure a protective yet not excessive inflammatory response.

The aims of this chapter were to determine the immunomodulation effect of *S. aureus* on co-infection of *B. cenocepacia*, to reveal the potential molecular mechanisms, as well as to characterise the nature of *S. aureus*-secreted immunomodulation molecules.

5.2 Suppression of *B. cenocepacia*-induced IL-8 production by *S. aureus* supernatants

Following co-stimulation with *B. cenocepacia* J2315 and *S. aureus* supernatants or heat-killed bacteria, the neutrophil chemoattractant IL-8 released by airway epithelial 16HBE cells was measured using ELISA. As shown in Chapter 3, *B. cenocepacia* J2315 is able to induce a robust IL-8 production. To optimise MOI (the ratio of bacteria to cells), 16 HBE cells were infected with an MOI ranging from 0.04 to 25 (Figure 5.1A) and all groups showed significant upregulation of IL-8, with a peak at an MOI of 5. Therefore, we chose an MOI of 5, for 24 hours, for the subsequent experiments.

Interestingly, although supernatants (LB medium) of MRSA252 or MSSA NCTC 6571 did not change IL-8 production compared to control, they significantly downregulated IL-8 induced by J2315 (23% and 31% less than J2315-treated group, respectively). To determine whether this effect is medium-specific, the same experiment was performed using supernatant from bacteria cultured in TSB (Figure 5.1C). In contrast, supernatants from *S. aureus* cultured in TSB maintained the level of IL-8 production with the presence or absence of J2315, which indicates that the anti-inflammatory effect is medium-specific. Similarly, J2315-induced IL-8 production did not change with supernatant of MRSA252 biofilm (cultured in LB) but decreased marginally with MSSA NCTC 6571 biofilm supernatant ($p<0.05$, Figure 5.1D).

Surprisingly, heat treatment (95°C, 10 minutes) did not reverse the anti-inflammatory property of *S. aureus* supernatants (Figure 5.1E), whereas heat-killed (100°C, 1 hour) *S. aureus* was unable to modulate IL-8 production (Figure 5.1F). To validate the results, the same experiments were done with a series of *S. aureus* isolates, either supernatants (Figure 5.2A) or heat-killed bacteria (Figure 5.2B). Supernatants from all the tested isolates downregulated the level of IL-8 to varying levels ($p<0.05$), whereas heat-killed bacteria had no effect on J2315-induced IL-8 production. These data indicate that anti-inflammatory substances exhibit in secreted products but not *S. aureus* cell surface structures. To confirm the suppressing effect is not due to cell damage, an MTT assay was used to assess cell viability (Figure 5.2C and D). Of note, supernatants of SH1000 and RN4220, which caused a doubling in IL-8 production (Figure 5.2A), significantly reduced cell viability. This indicates the correlation between pro-inflammatory secreted products and their virulence. In contrast, heat-killed *S. aureus* did not affect cell viability.

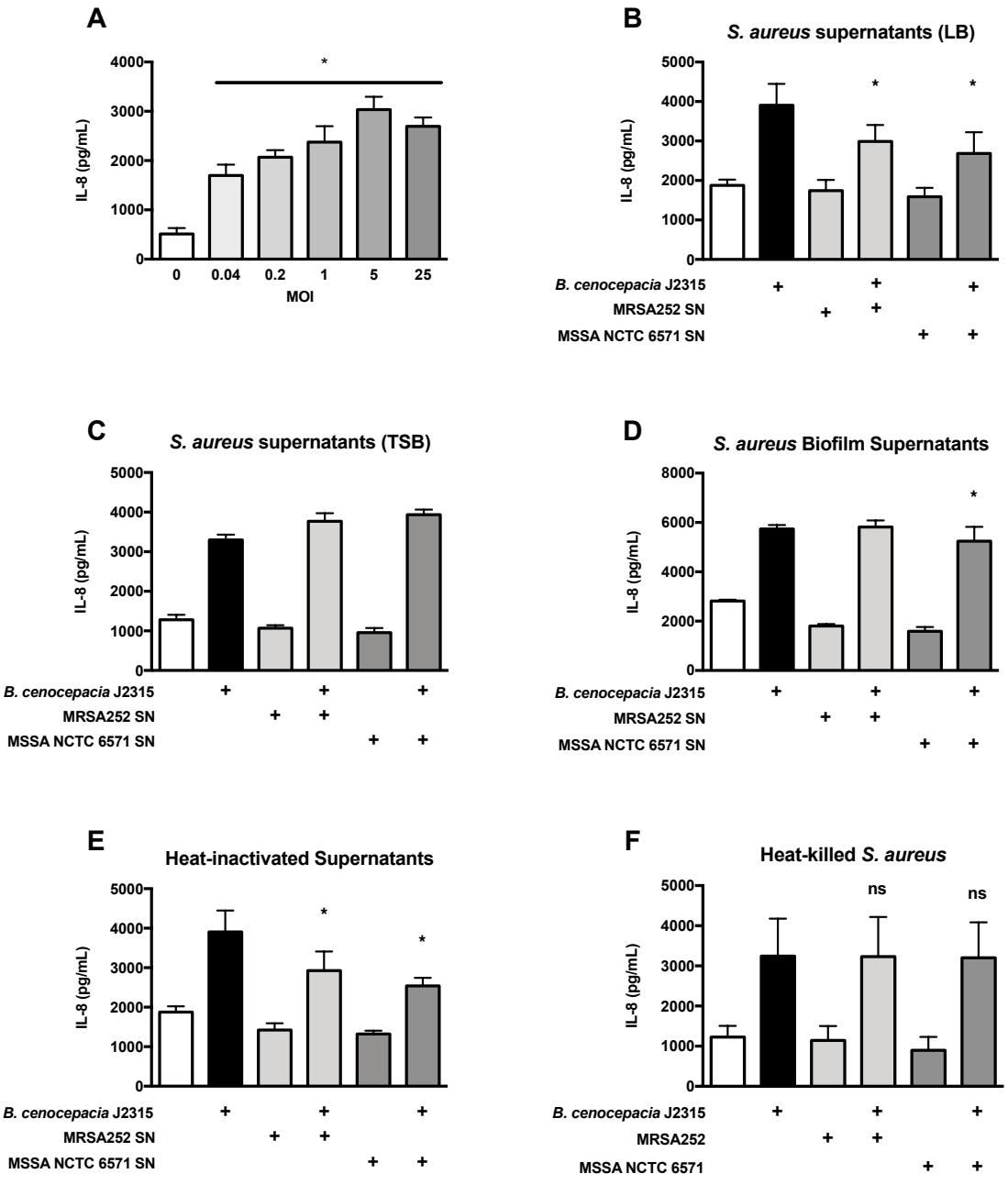

Figure 5.1 The anti-inflammatory effect of *S. aureus* on *B. cenocepacia* J2315-induced IL-8.

(A) Dose response of 16HBE cells to *B. cenocepacia* J2315 live bacteria for 24 hours. 16HBE cells were treated with *B. cenocepacia* J2315 at an MOI of 5, in the presence or absence of **(B)** *S. aureus* supernatants 10% (v/v in LB), **(C)** 10% (v/v in TSB), **(D)** 10% biofilm supernatants (v/v in LB), **(E)** heat-inactivated supernatants 10% (v/v in LB), **(F)** heat-killed *S. aureus* (5 MOI) for 24 hours. The control group was treated with 10% (v/v) LB in B, D and E; 10% (v/v) TSB in C. IL-8 release was measured by ELISA. Data represent mean ± SEM from 3 independent experiments performed in duplicate. Significance was compared to **(A)** the control or **(B-F)** J2315-treated group using 2-way ANOVA followed by Dunnett's multiple comparisons test.

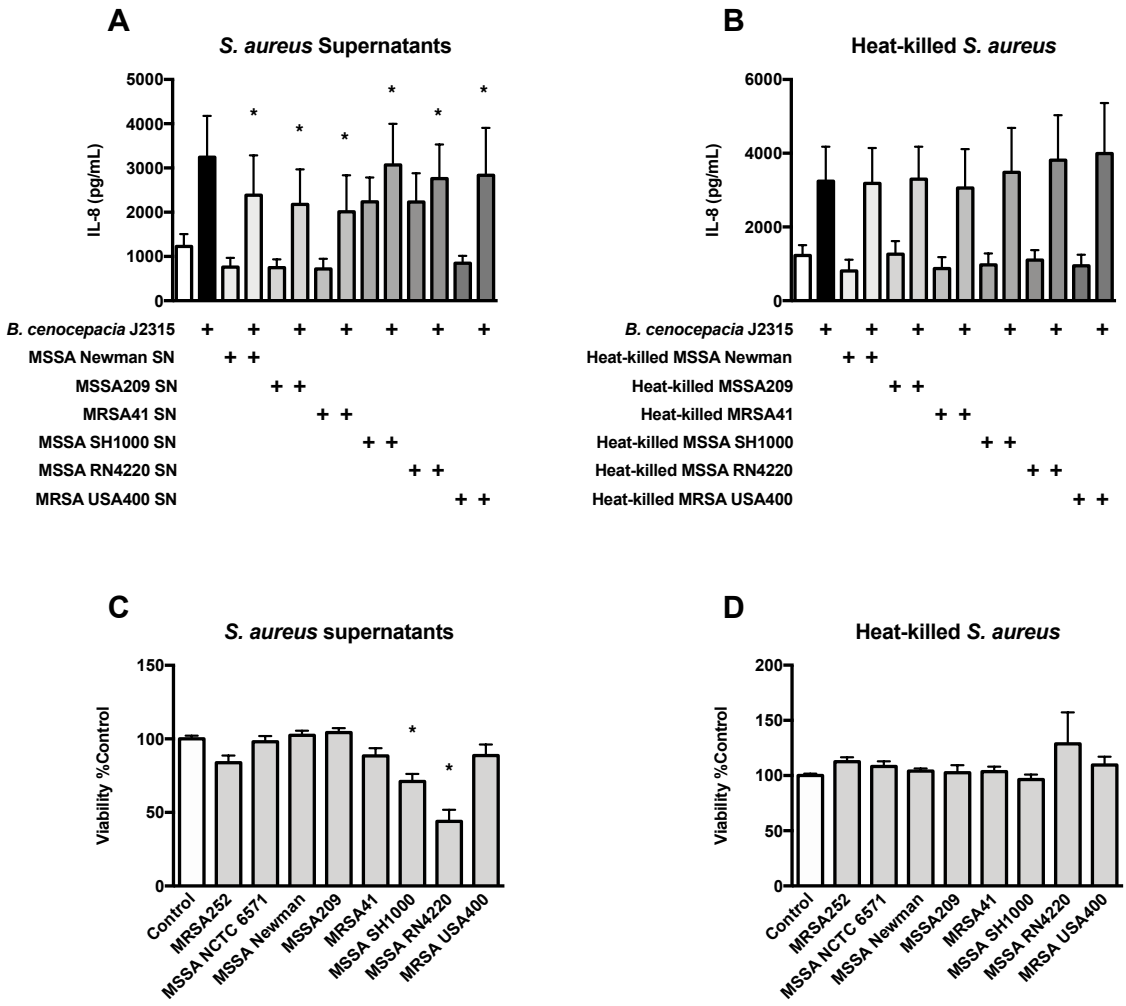

Figure 5.2 IL-8 response to co-stimulation with *B. cenocepacia* J2315 and different *S. aureus* supernatants or heat-killed *S. aureus*.

16HBE cells were stimulated with *B. cenocepacia* J2315 (5 MOI), in the presence or absence of **(A**) *S. aureus* supernatants (10% v/v) for 24 hours, or **(B)** heat-killed *S. aureus* (5 MOI) for 24 hours. The control group was treated with 10% (v/v) LB in A. IL-8 release was measured by ELISA. Data represent mean ± SEM from 3 independent experiments performed in duplicate. Significance compared to J2315-treated group using 2-way ANOVA followed by Dunnett's multiple comparisons test. **(C, D)** MTT assay was used to assess cellular cytotoxicity caused by *S. aureus* supernatants and heat-killed *S. aureus*. Results are expressed as percent of the control, representing mean ± SEM (n=3). Significance compared to the control using 1-way ANOVA followed by Dunnett's multiple comparisons test.

In order to study the modulation on host signalling by this co-stimulation model, 16HBE cells were treated with *B. cenocepacia* J2315 (50 MOI) and 25% (v/v) MSSA NCTC 6571 supernatant for 5 minutes (Figure 5.3A and B). J2315 caused a marginal downregulation on phosphorylation of p38 and Erk, whilst *S. aureus* supernatant caused a rapid phosphorylation, particularly with Erk. Co-stimulation led to activation of p38 and Erk, which likely results from the effect *of S. aureus*. Following 1 hour pretreatment of *S. aureus* supernatant (25% v/v), 16HBE cells were infected with J2315 (50 MOI) for 5 minutes (Figure 5.3C, D and E). J2315 and *S. aureus* induced a marginal increase in phosphorylation of p38, whilst dramatically increasing phosphorylation of Erk and IκBα, which indicates *S. aureus* supernatant at a higher concentration is able to induce persistent phosphorylation of Erk and IκBα. However, when co-stimulation extended to 2 hours some trends became visible (Figure 5.4), although not achieving statistical significance. *B. cenocepacia* J2315 caused activation of Akt, p38, Erk and p65, whereas *S. aureus* did not change any tested signalling. It is worth noting that unlike MRSA252 supernatant, MSSA NCTC 6571 supernatant abolished phosphorylation of p38, Erk and p65 caused by *B. cenocepacia* J2315. Therefore, these findings suggest that *S. aureus* supernatants may suppress the pro-inflammatory effect of *B. cenocepacia* by blocking p38, Erk and NF-κB.

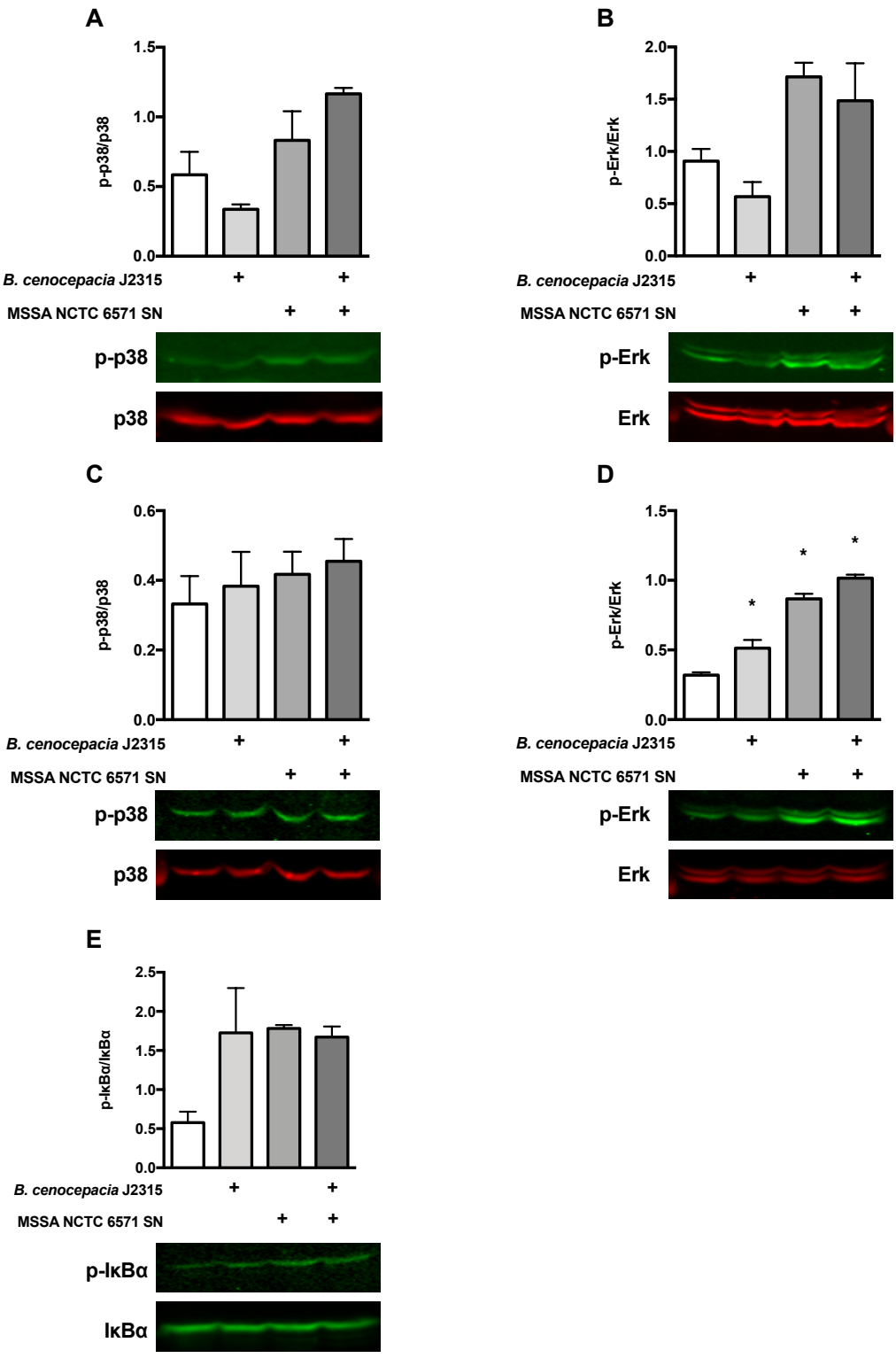

Figure 5.3 MAPK response to co-stimulation with *B. cenocepacia* J2315 and MSSA NCTC 6571 supernatant.

(A-B) 16HBE cells were treated with LB (25% v/v) as control, J2315 (50 MOI), *S. aureus* supernatant (25% v/v) for 5 minutes. **(C-E)** After 1 hour pretreatment with *S. aureus* supernatant (25% v/v), 16HBE cells were infected with J2315 (50 MOI). The control was treated with 25% (v/v) LB for 1 hour. Phosphorylation of p38, Erk and IκBα of cell lysates were measured using western blot and normalised against total p38, Erk and IκBα, respectively. Data are shown as mean ± SEM from three independent experiments. *, $p<0.05$ compared with the control using one-way repeated measures ANOVA following Dunnett's multiple comparisons test.

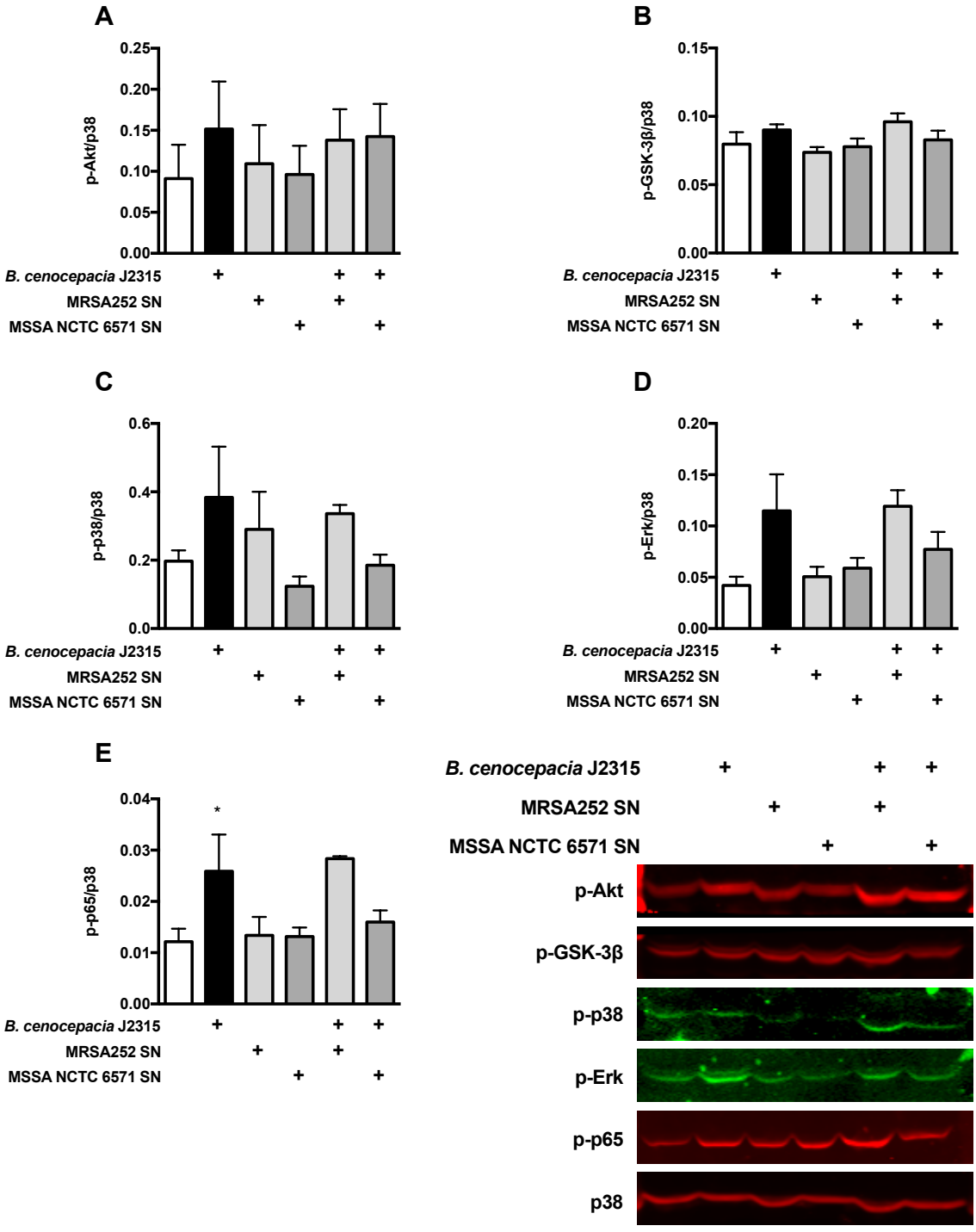

Figure 5.4 PI3K, MAPK and NF-κB response to co-stimulation with *B. cenocepacia* J2315 and MRSA252 or MSSA NCTC 6571 supernatant.

16HBE cells were treated with LB (10% v/v) as the control, J2315 (5 MOI), with the presence or absence of *S. aureus* supernatant (10% v/v) for 2 hours. Phosphorylation of Akt **(A)**, GSK-3β **(B)**, p38 **(C)**, Erk **(D)** and p65 **(E)** of cell lysates were measured using western blot and normalised against total p38. **(F)** Western blot analysis for levels of phosphorylated Akt, phosphorylated GSK-3β, phosphorylated p38, phosphorylated Erk, phosphorylated p65, and p38 in cell lysate following S. aureus supernatants and *B. cenocepacia* J2315 co-stimulation. Protein bands representative of three independent experiments. Data are shown as mean ± SEM. *, $p<0.05$ compared with the control using one-way repeated measures ANOVA following Dunnett's multiple comparisons test.

5.3 Characterisation of anti-inflammatory factors in *S. aureus* supernatants

It has been confirmed that the anti-inflammatory factors in *S. aureus* supernatant are heat-stable and not associated with cell wall structures (Figure 5.1E-F). To determine the nature of the effectors, *S. aureus* supernatants were digested with the broad-spectrum serine protease, PK or heat-inactivated PK (Figure 5.5A). Co-stimulation with *B. cenocepacia* J2315 and PK-treated supernatants did not restore IL-8 production to the level as J2315-treated group; on the contrary, IL-8 production dropped to the same level of the control. This indicates that the effectors are not proteinaceous. To assess the approximate molecular weight, *S. aureus* supernatants were separated into different molecular cut-off fractions (Figure 5.5B). Although there was a weak positive control response in this series of experiments, supernatants <3 kDa still abolished IL-8 production back to the same level as the control, which indicates the anti-inflammatory molecules are small. In order to elucidate whether TLR is exclusively involved in *S. aureus*-induced anti-inflammatory response, 16HBE cells were stimulated with up to 10 ng/mL IL-1β. However, IL-1β only induced half the amount of IL-8 induced by *B. cenocepacia* J2315. In addition, IL-1β / *S. aureus* supernatants co-stimulation caused a marginal increase rather than a decrease (Figure 5.5C). Therefore, a further experiment was done by co-stimulating with IL-1β and TNFα, which resulted in a similar level of IL-8 compared to J2315-treated group (Figure 5.5D). However, no effect of *S. aureus* supernatants was observed on IL-1β / TNFα-induced IL-8 production, indicating IL-1R and TNFR are not associated with this process and the suppressive effect does not appear to be a non-selective downregulation of IL-8 production.

Taken together, the anti-inflammatory molecules in *S. aureus* supernatants are characterised as small, heat-resistant, non-proteinaceous, non-cell wall components.

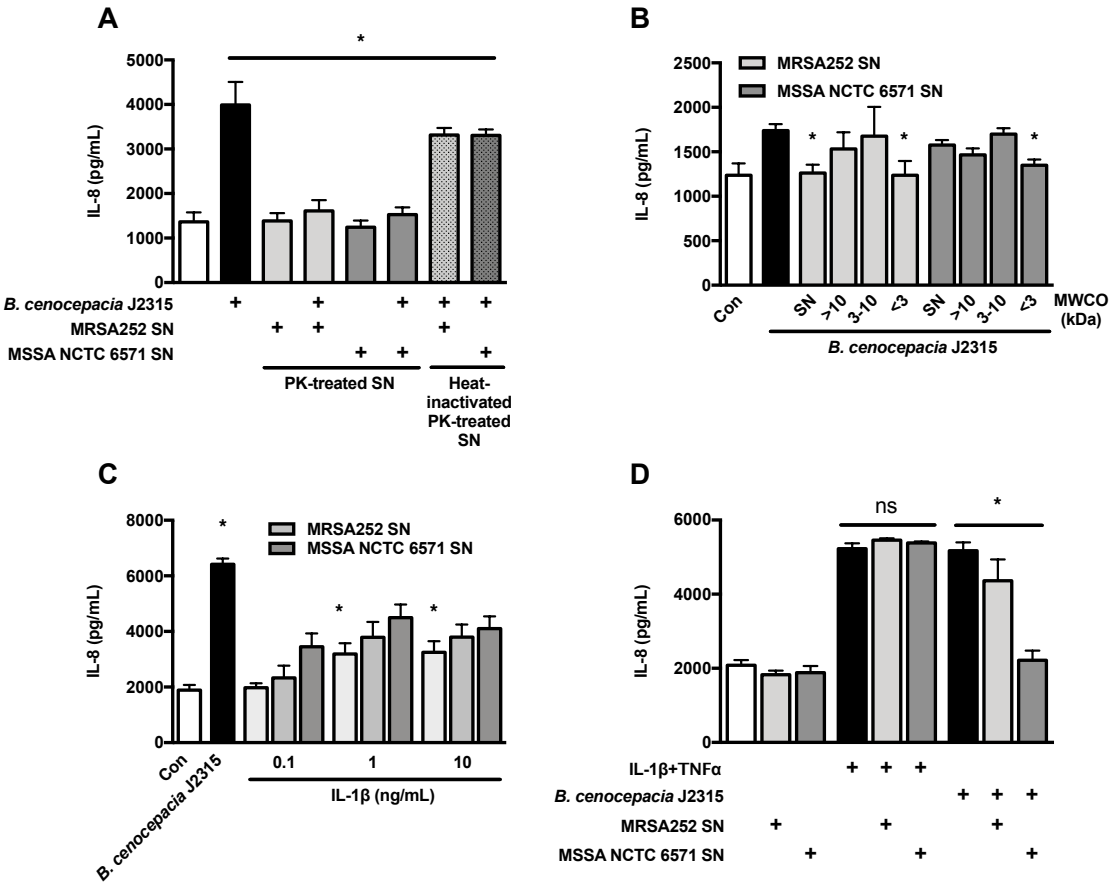

Figure 5.5 Characterisation of anti-inflammatory effectors in *S. aureus* supernatants.

(A) *S. aureus* supernatants were treated with immobilised proteinase K for 1 hour at 37°C, or treated with heat-inactivated proteinase K (99°C for 1 hour) as the positive control. 16HBE cells were co-stimulated with *B. cenocepacia* J2315 (5 MOI) and proteinase K-treated *S. aureus* supernatants (10% v/v) for 24 hours.

(B) *S. aureus* supernatants were filtered through selective membranes. 16HBE cells were treated with *B. cenocepacia* J2315 (5 MOI) and *S. aureus* supernatant fractions containing metabolites of >10 kDa, >3 kDa and <3 kDa for 24 hours. IL-8 release was measured by ELISA. Data represent mean ± SEM from 3 independent experiments performed in duplicate. Significance compared to J2315-treated group using 2-way ANOVA followed by Dunnett's multiple comparisons test.

(C) After 1 hour pretreatment of *S. aureus* supernatants (10% v/v), 16HBE cells were stimulated with increasing dose of IL-1β for 24 hours. Data represent mean ± SEM from 3 independent experiments performed in duplicate. Significance was compared to the control using 2-way ANOVA followed by Dunnett's multiple comparisons test.

(D) 16HBE cells were co-stimulated with IL-1β (1 ng/mL) / TNFα (10 ng/mL) and *S. aureus* supernatants (10% v/v) for 24 hours. *B. cenocepacia* J2315 (5 MOI) was the positive control. Data represent mean ± SEM from 3 independent experiments performed in duplicate. *, $p<0.05$ compared to IL-1β / TNFα-treated group or J2315-treated group using 2-way ANOVA followed by Dunnett's multiple comparisons test.

5.4 16HBE cells are hypo-responsive to Pam_3CSK4, LPS but not flagellin

As a Gram-negative bacterium with flagella, *B. cenocepacia* J2315 potentially binds to and activates TLR2, TLR4 and TLR5 (de C Ventura et al., 2008; West et al., 2008). To determine which TLR is involved in *B. cenocepacia / S. aureus* polymicrobial infection setting, 16HBE cells were co-stimulated with TLR2, TLR4 and TLR5 agonists, Pam_3CSK4, LPS and flagellin, respectively, and with the presence or absence of MSSA NCTC 6571 supernatant (Figure 5.6). None of the agonists induced IL-8 production as well as J2315, and only flagellin triggered a dose-dependent, significantly upregulated IL-8 response, indicating the pro-inflammatory property of J2315 is associated with multiple virulence factors. Moreover, with all agonists a marginal drop of the IL-8 response was seen with the presence of *S. aureus* supernatant.

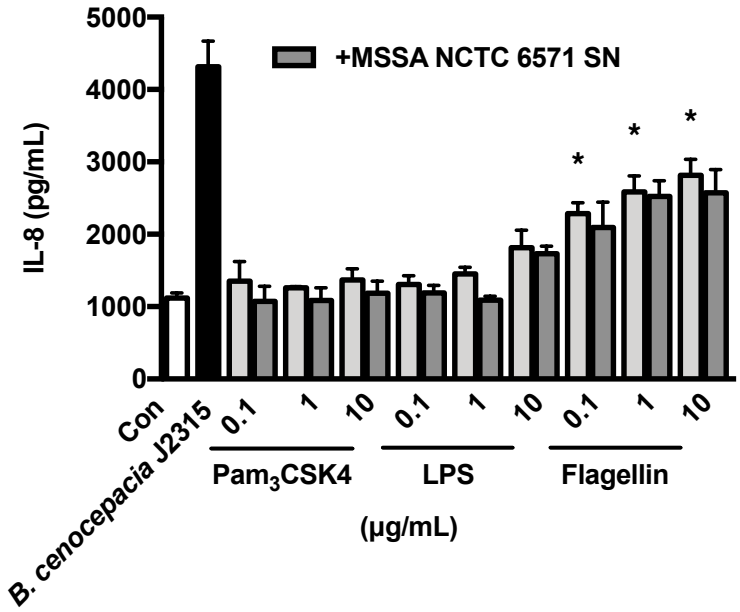

Figure 5.6 Pro-inflammatory response to TLR2, 4, or 5 agonists and *S. aureus* supernatant.

16HBE cells were co-stimulated with increasing dose of Pam_3CSK4, LPS, flagellin and MSSA NCTC 6571 supernatant (10% v/v) for 24 hours. *B. cenocepacia* J2315 (5 MOI) was the positive control. IL-8 release was measured by ELISA. Data represent mean ± SEM from 3 independent experiments performed in duplicate. *, $p<0.05$ compared to control using 2-way ANOVA followed by Dunnett's multiple comparisons test.

To investigate host signalling responses to Pam_3CSK4 and flagellin, the time course of the response was assessed. No effect was seen with Pam_3CSK4 (10 μg/mL) up to 60 minutes (Figure 5.7), whereas flagellin (1 μg/mL) caused a transient phosphorylation of Akt, GSK-3β, p38, Erk and p65 between 15 and 30 minutes (Figure 5.8), which is consistent with the IL-8 response. This indicates that flagellin might play a major role in the *B. cenocepacia* induced pro-inflammatory response.

Following pretreatment with the TLR4 antagonist LPS-RS, stimulation with J2315 showed significant downregulation in IL-8 production (14% decrease compared to the J2315-treated group), to the same level as *S. aureus* supernatants (Figure 5.9A). In contrast, co-stimulation with TLR5 antagonist hTLR5fc and J2315 did not have any effect compared to the J2315-treated group (Figure 5.9B).

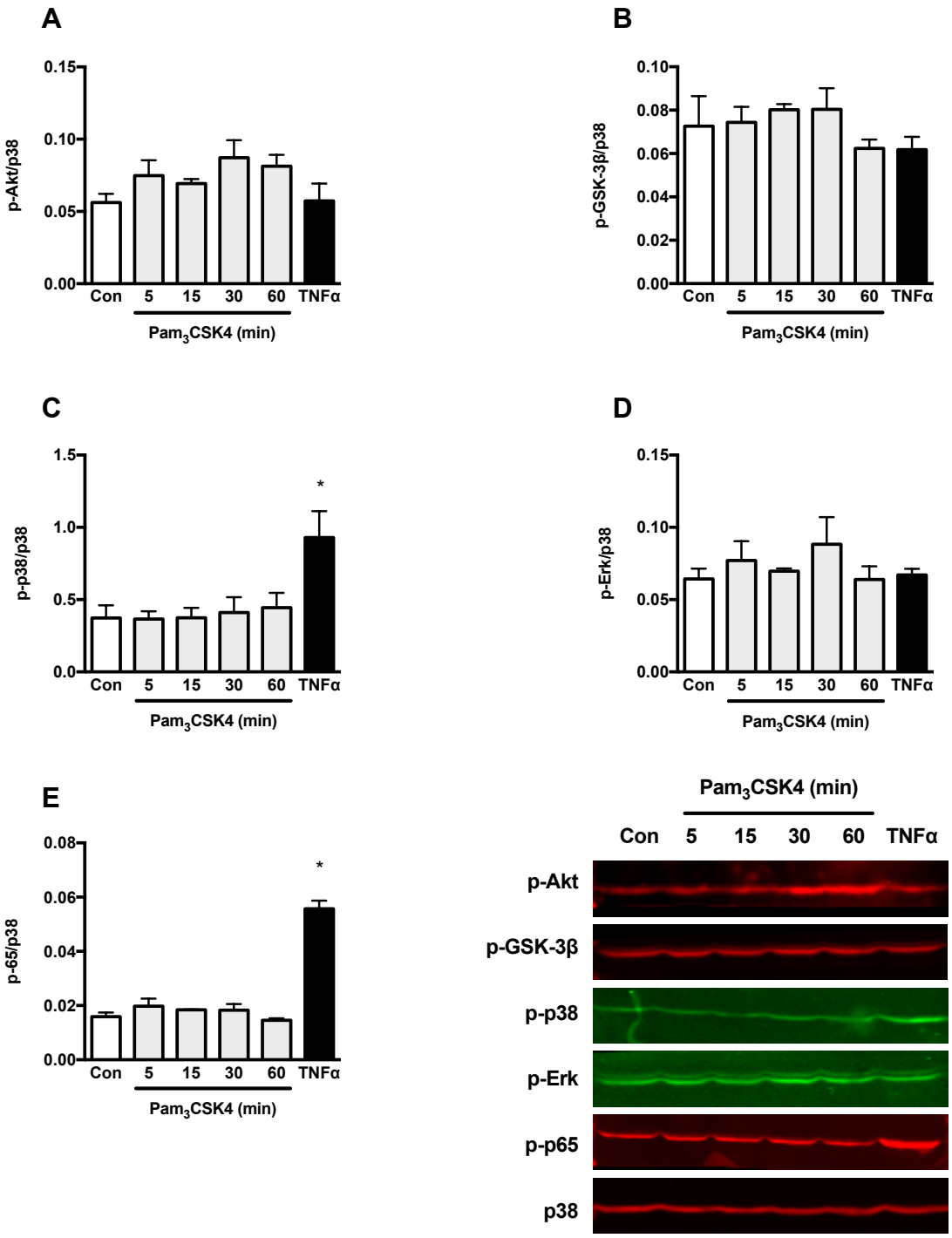

Figure 5.7 Time course of Pam_3CSK4 on PI3K, MAPK and NF-κB activation.

16HBE cells were treated with Pam_3CSK4 (10 μg/mL) for 5-60 minutes, TNFα (20 ng/mL, 5 minutes) as the positive control. Phosphorylation of Akt **(A)**, GSK-3β **(B)**, p38 **(C)**, Erk **(D)** and p65 **(E)** of cell lysates were measured using western blot and normalised against total p38. **(F)** Western blot analysis for levels of phosphorylated Akt, phosphorylated GSK-3β, phosphorylated p38, phosphorylated Erk, phosphorylated p65, and p38 in cell lysate folowing Pam_3CSK4 stimulation. Protein bands representative of three independent experiments. Data are shown as mean ± SEM. *, $p<0.05$ compared with control using one-way repeated measures ANOVA following Dunnet's multiple comparisons test.

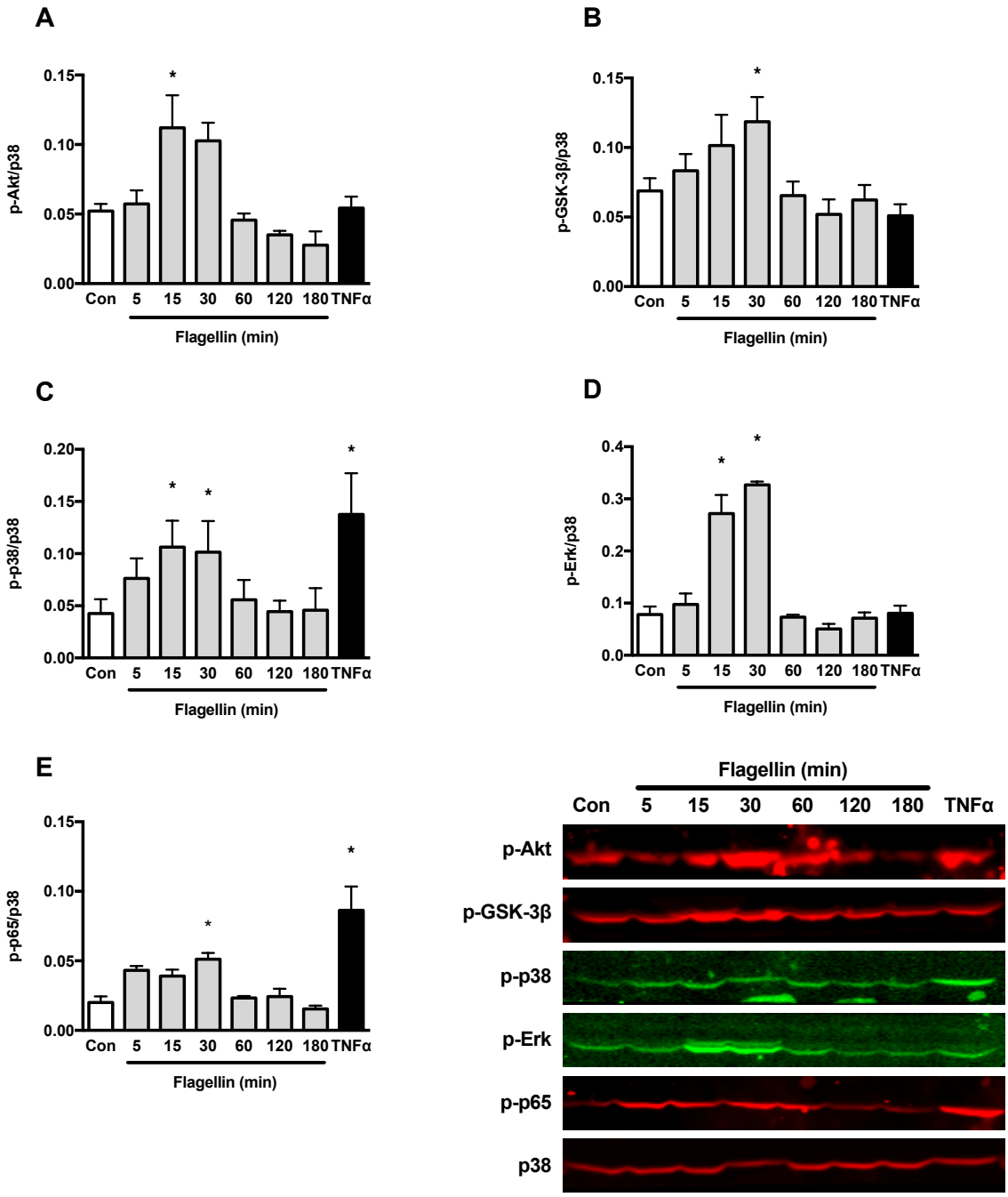

Figure 5.8 Time course of flagellin on PI3K, MAPK and NF-κB activation.

16HBE cells were treated with flagellin (1 μg/mL) for 5-180 minutes, TNFα (20 ng/mL, 5 minutes) as the positive control. Phosphorylation of Akt **(A)**, GSK-3β **(B)**, p38 **(C)**, Erk **(D)** and p65 **(E)** of cell lysates were measured using western blot and normalised against total p38. **(F)** Western blot analysis for levels of phosphorylated Akt, phosphorylated GSK-3β, phosphorylated p38, phosphorylated Erk, phosphorylated p65, and p38 in cell lysate folowing flagellin stimulation. Data are shown as mean ± SEM from three independent experiments. *, $p<0.05$ compared with control using one-way repeated measures ANOVA following Dunnet's multiple comparisons test.

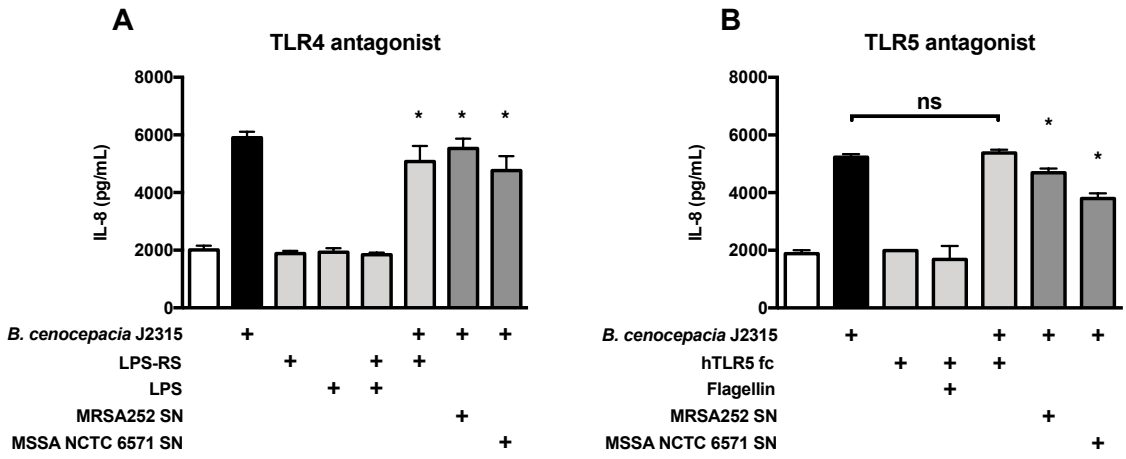

Figure 5.9 TLR4 antagonist LPS-RS inhibits *B. cenocepacia* J2315-induced IL-8 production but not TLR5 antagonist hTLR5 fc.

(A) Following pretreatment with TLR4 antagonist LPS-RS (2 µg/mL) for 1 hour, 16HBE cells were stimulated with LPS (10 µg/mL) or *B. cenocepacia* J2315 (5 MOI) for 24 hours. Cells were also co-stimulated with *S. aureus* supernatants (10% v/v,1 hour pretreatment) and *B. cenocepacia* J2315 (5 MOI) for 24 hours as positive controls. **(B)** After 24 hours pretreatment with TLR5 antagonist hTLR5-fc (100 ng/mL), 16HBE cells were stimulated with TLR5 agonist flagellin (1 µg/mL) or *B. cenocepacia* J2315 (5 MOI) for 24 hours. Cells were co-stimulated with *S. aureus* supernatants (10% v/v,1 hour pretreatment) and *B. cenocepacia* J2315 (5 MOI) for 24 hours as positive controls. IL-8 release was measured by ELISA. Data represent mean ± SEM from 3 independent experiments performed in duplicate. *, $p<0.05$ compared to *B. cenocepacia*-J2315 treated group using 2-way ANOVA followed by Dunnett's multiple comparisons test.

5.5 Discussion

Several lines of evidence indicate that *S. aureus* induces an anti-inflammatory response to facilitate persistence inside the host. For example, the secreted protein SSL3 blocks TLR2 activity and thereby inhibits IL-8 production *in vitro* (Bardoel et al., 2012), as well as antagonising TLR2 function as a virulence factor during *in vivo* infection of mice (Koymans et al., 2017). Additionally, *S. aureus*-induced IL-10 production enhances persistence by hindering local pro-inflammatory responses in an acute murine skin infection model (Leech et al., 2017). Because of the large reservoir of immunomodulatory effectors (Thammavongsa et al., 2015), multi-drug resistance, and biofilm formation, *S. aureus* is a major threat to human health in infectious diseases.

Extensive studies have focused on the interactions between *S. aureus* and *P. aeruginosa* as they are two prevalent pathogens involved in CF lung infections. However, *S. aureus*/Bcc interactions and their impact on host response have not been assessed before. Herein, we demonstrate that in the context of *S. aureus / B. cenocepacia* co-infection, supernatants from *S. aureus* isolates dampen *B. cenocepacia* live bacteria-induced IL-8 production in airway epithelial cells, indicating that *S. aureus* releases anti-inflammatory compounds into the broth. Interestingly, all the supernatants from *S. aureus* isolates block J2315-induced IL-8 production to different levels ($p<0.05$). Hyperinflammatory isolates such as SH1000, RN4220 and USA400, where the live bacteria induced strong IL-8 production (Figure 3.3A), exhibit inhibitory effect on IL-8 production as well. This indicates the components may be widely expressed in *S. aureus*.

In agreement with our findings, Chekabab et al. (2015) demonstrated that *S. aureus* supernatants suppress *P. aeruginosa* supernatant-induced cytokine (IL-8 and CXCL-2) production through inhibiting TLR1/2 and TLR4, but not TLR5. However, the inhibition effect is medium-specific, as it presents with LB-cultured supernatant but not TSB-cultured supernatant, which is in parallel with our results. LB and TSB are rich media, commonly used to culture aerobic microorganisms such as *S. aureus*. Compared to TSB, LB has simple ingredients and is not buffered. Unfortunately, we are unable to confirm which medium reflects the physiological condition of the airway milieu. Several studies have applied synthetic cystic fibrosis sputum medium (Kumar and Cardona, 2016) or artificial sputum medium (Fung et al., 2010) to resemble the sputum from CF patients and observed changes in gene expression responsible for virulence. Since differences in compositions of culture media may change metabolism and subsequently affect modulation of metabolites on host response, *S. aureus* should be cultured in a medium that reflects the nutrition of the infected airway. Nevertheless, *S. aureus* grows poorly in

synthetic CF sputum medium and thus is not possible to compare the differences of *S. aureus* supernatant cultured in synthetic CF sputum medium and LB (Bernier et al., 2016).

S. aureus biofilm supernatants are able to impair macrophage phagocytosis (Hanke and Kielian, 2012) and *S. aureus* biofilms programme macrophages to the alternative activated M2 phenotype (Thurlow et al., 2011), It was unexpected that in our results *S. aureus* biofilm supernatants had very little effect on J2315-induced IL-8, except a marginal downregulation by MSSA NCTC 6571 biofilm supernatant ($p<0.05$). Supernatants from planktonic and biofilm *S. aureus* contain different features (such as proteases in biofilm supernatant) which result in different signalling activation and cytokine production. Compared to the planktonic counterparts, biofilm supernatants cause apoptosis, downregulate phosphorylation of p38 and JNK, upregulate phosphorylation of Erk, and induce higher level of cytokines at 4 hours but lower level at 24 hours post-stimulation in human keratinocytes (Secor et al., 2011). Differential host responses directed towards planktonic and biofilm supernatants indicate that *S. aureus* modifies its expression of virulence factors at different stages of infection. Brady et al. (2018) developed epicutaneous (planktonic) and subcutaneous mesh (biofilm) *S. aureus* infection models in mice and compared cytokine production at 7 days and 21 days. Although the day 7 biofilm model caused higher levels of CXCL1, CXCL2, IL-1α and IL17A production compared to the planktonic counterpart, the host response was ineffective and insufficient for microbial clearance. On day 21, *S. aureus* biofilm shifted to a dormant state, which results in *S. aureus* being less detectable to the immune system as shown by a decrease in cytokines released (Brady et al., 2018).

In further experiments, the effects of heat-treatment, heat-killed *S. aureus*, proteinase K-treatment, and different molecular weight cut-off fractions were examined in order to determine the nature of the anti-inflammatory compounds in *S. aureus* supernatants. The compounds were characterised as small (<3 kDa), heat-resistant, non-proteinaceous, and non-cell wall components, in agreement with previous work (Chekabab et al., 2015). When co-stimulated with J2315, IL-8 production elicited by proteinase K-treated *S. aureus* supernatants showed a 50% decrease compared to deactivated proteinase K-treated *S. aureus* supernatants. This indicates that the anti-inflammatory components is less likely to be peptides or proteins. The decrease may result from pro-inflammatory components in *S. aureus* supernatants spontaneously degraded by proteinase K. Apart from releasing immunosuppression modulators, *S. aureus* produces multiple virulence factors that have pro-inflammatory effects. For example, MRSA-derived small, heat- and protease-resistant molecules induce nitric oxide in human sinonasal epithelial cells (Carey et al., 2015). The level of nitric oxide and inflammation status are positively correlated as nitric oxide plays

a critical role in microbial killing, wound healing as well as facilitating chemokine production (Trifilieff et al., 2000). In contrast with our observations, *S. aureus* supernatants are reported to induce a stronger magnitude of cytokine, chemokine and prostaglandin E_2 expression than live bacteria (Moreilhon et al., 2005). These findings suggest that *S. aureus* as a prevalent pathogen modulates the immune response to benefit colonisation and infections.

Inhibition of IL-8 production by *S. aureus* supernatants has been previously reported in TNFα-treated HUVEC cells (Tajima et al., 2006). The inhibitory effect of supernatants is also abolished by proteinase K or trypsin treatment but, in contrast with our findings, is sustained after heat-treatment. Thus, the anti-inflammatory component is defined as heat-stable protein. Of note, *S. aureus* supernatants are treated with proteinase K and trypsin, respectively; however, they did not describe whether the final supernatants contain proteinase K. Serine proteases such as trypsin activate proteinase-activated receptor-2 which subsequently activates NF-κB (Buddenkotte et al., 2005). Although there is no evidence showing that proteinase K causes activation of NF-κB, it may be that stimulating HUVEC cells with residual proteinase K (10 μg/mL according to their method) initiated an inflammatory response that masked the *S. aureus* inhibitory activity.

Immunosuppression is also observed with commensal *S. salivarius*, which dwells in the oral and nasopharyngeal cavity. *S. salivarius* secretes small heat-resistant proteins which hinder p65 nuclear translocation and subsequently IL-8 production, both under baseline condition and on co-stimulation with different stimuli (TNFα, IL-1β, *P. aeruginosa*, flagellin, LL-37) (Cosseau et al., 2008; Kaci et al., 2014; 2011). The abilities of commensal bacteria to cause a low pro-inflammatory response, induce an anti-inflammatory respons and regulate gene expression involved in airway epithelial tight junctions contribute to immune tolerance and homeostasis in the mucosal barrier. Our results showed that the inhibitory effect of *S. aureus* secreted products was TLR-specific, as *S. aureus* supernatants did not affect IL-1β/TNFα-induced IL-8 production. In contrast, *S. aureus* supernatants suppress TNFα-induced IL-8 production in the HUVEC model. However, the supernatants are obtained using a different method (incubated for 1 hour in mammalian cell culture medium) which may elicit different metabolites (Tajima et al., 2006). Overall, *S. aureus* may induce anti-inflammatory responses to prevent phagocytosis and hinder the adaptive immune response. Thus, it is critical to elucidate the immune evasion mechanisms used by *S. aureus* to design novel treatments.

Following co-stimulation with *B. cenocepacia* J2315 and *S. aureus* supernatant, we assessed activation of PI3K, MAPK and NF-κB and found that MSSA supernatant

abolished phosphorylation of p38, Erk and p65 caused by J2315. MRSA252 supernatant did not affect either baseline or J2315-induced phosphorylation, which correlates with its less downregulation effect on IL-8 production compared to MSSA NCTC 6571. Our results are in partial agreement with the results by Chekabab et al., (2015) as they showed *S. aureus* supernatants block NF-κB activation but not p38. However, Chekabab et al. (2015) stimulated airway epithelial Beas-2B cells with synthetic TLR2/1 agonist Pam_3Csk4, whereas *B. cenocepacia* contains more complicated ligands. Additionally, the downstream signalling cascades engaged in the anti-inflammatory response may be cell type-specific. Nasal *S. aureus* isolates utilise PI3K and Erk signalling cascades to induce IL-10 production whereas they induce TNFα in a p38-dependent pathway in human PBMC (Peres et al., 2015).This suggests that through expressing unique ligands in the process of infection, *S. aureus* manipulates different signalling cascades to initiate immune tolerance or phagocytosis.

Chekabab et al. (2015) suggest that the inhibitory effect of *S. aureus* supernatant is through TLR signalling. In order to determine which TLR signalling was modulated by *S. aureus*-secreted products, first we stimulated 16HBE cells with TLR2/1, TLR4 and TLR5 agonists, respectively. Pam_3CSK4, LPS and flagellin caused 22%, 62% and 151% increases in IL-8 production at the highest dose (10 μg/mL) compared to the control. The result was unexpected, as in Beas-2B cells Pam_3CSK4 was found to be the most efficacious IL-8 inducer, LPS showed a dose-dependent manner of inducing IL-8 and flagellin did not affect IL-8 production (Chekabab et al., 2015). The hypo-responsiveness to TLR2/1 and TLR4 agonists may result from low expression and location of the receptors and the accessory molecules, as in polarised primary human airway epithelial cells TLR2 is mainly expressed on the basolateral surface and TLR4 is weakly expressed on the apical surface (Ioannidis et al., 2013). For example, airway epithelial Beas-2B cells display lower sensitivity to *S. aureus* and TLR2 agonists compared to Gram-negative bacteria in terms of IL-8 production, which is associated with low expression of TLR2 and missing expression of the co-receptor CD36; whereas monocytes exhibit a robust TNFα response to both Gram positive and negative bacteria (Mayer et al., 2007). This indicates that mucosal epithelia may use this mechanism to prevent an excessive inflammatory response and maintain homeostasis. In contrast, *B. cenocepacia* J2315 can induce TLR2 and TLR4, but not TLR5 expression in Beas-2B cells, whereas only TLR5 is required for a pro-inflammatory response (de C Ventura et al., 2008).

Further we assessed the effect of Pam_3CSK4 and flagellin on signalling cascades. It is not surprising that Pam_3CSK4 did not affect PI3K, MAPK and NF-κB. In agreement with our result, Pam_3CSK4 is able to trigger marginal phosphorylation of p38 but not NF-κB

activity and IL-8 production in human airway epithelial cells (Winder et al., 2009). Flagellin has been reported to activate phosphorylation of p38 and p65 in airway epithelial Calu-3 cells (Illek et al., 2008), phosphorylation of Akt in human colonic epithelial cells (Sang et al., 2006), and phosphorylation of p38, Erk, JNK and IκBα in mouse bone marrow-derived dendritic cells (Feuillet et al., 2006). In our system, airway epithelial cells were most sensitive to flagellin, as flagellin activated several signalling pathways and induced a strong IL-8 response. In the following experiment, we pretreated the cells with TLR4 and TLR5 antagonists, respectively, followed by stimulation with TLR agonists or J2315. Compared to J2315-treated cells, only the TLR4 antagonist caused a decrease in IL-8 production. However, the magnitude of reduction was similar to *S. aureus* supernatants. This suggests that *S. aureus*-secreted products may block TLR4 and subsequently suppress the *B. cenocepacia*-induced pro-inflammatory response. Recently, a family of small (~20-25 amino acids) secreted peptides, namely PSMs are able to bind to TLR4 without activation in THP-1 cells (Chu et al., 2018). PSM α1-α3 suppress pro-inflammatory cytokines production induced by high mobility group box-1 protein (belongs to the danger-associated molecular pattern family) by subverting TLR4 signalling. However, although heat stability of these factors is unclear, according to our results the anti-inflammatory factors may not be peptide/protein. Thus, further characterisation needs to identify the Staphylococcal factors.

5.6 Conclusions

In this chapter, we demonstrate that *S. aureus* isolates produce immunomodulatory factors that subvert the *B. cenocepacia*-induced inflammatory response in airway epithelial cells. The factors are characterised as small, heat-stable, non-proteinaceous, non-cell wall components. Additionally, we demonstrate *S. aureus* subverts the *B. cenocepacia*-induced inflammatory response by attenuation of TLR4-mediated p38, Erk and NF-κB siganlling cascades. Further experiments are needed to identify the active factors.

Chapter 6 General discussion

6.1 Host responses to monomicrobial infections

The innate immunity protects the lower respiratory tract and controls its homeostasis. Intensity and duration of the immune response, along with pathogenesis of the pathogens, determine the clinical outcome (Strobel et al., 2016). The host-pathogen interactions in the scenario of polymicrobial infections have been acknowledged with increasing frequency, yet still require intensive investigation (Bragonzi et al., 2012; Dai et al., 2018; Flight et al., 2015; Sibley et al., 2008). This thesis began by assessing pathogenesis of individual bacterial pathogens that belong to *S. aureus* and Bcc species, and the effect on host signalling cascades and pro-inflammatory responses. Upon recognition of incoming microorganisms, certain signalling pathways are activated to initiate the expression of pro-inflammatory cytokines and chemokines as key events to conduct and amplify the immune response (Weitnauer et al., 2016; Whitsett and Alenghat, 2015).

To enhance the potential of pathogenesis, multiple virulence factors are required at different stages of infection (Foster et al., 2014; Kim et al., 2010). Thus, microbial invasion was assessed by viable count using airway epithelial cells grown on plastic. Airway epithelial cells are vulnerable to *S. aureus* invasion, and might be killed by virulent isolates (Strobel et al., 2016). We observed that epithelial cells accumulated higher amounts of *S. aureus* on the cell membrane compared to clinical Bcc isolates, whereas the latter was taken up more readily. To assess invasion on intact polarised epithelial monolayers, airway epithelial cells were cultured on transmembrane inserts, infected apically with live bacteria, and the integrity was monitored using TEER. In agreement with a previous study, our isolates of interest, MRSA252 and *B. multivorans* 13243 both breached polarised epithelial monolayers in 24 h. By disturbing the actin cytoskeleton, *S. aureus* or *B. multivorans* invade the epithelium barrier (Schwab et al., 2002; Soong et al., 2011). *B. multivorans* also leads to extensive cell death (Schwab et al., 2002), which might explain why *B. multivorans* caused higher level of FITC-dextran flux compared to *S. aureus*. This indicates that *B. multivorans* resulted in a higher barrier permeability than *S. aureus*.

As PI3K regulates cytoskeleton rearrangement (Gessain et al., 2015), we assessed invasion by treating airway epithelial cells with pharmacological PI3K inhibitors prior to live bacteria exposure. In line with a previous study (Kierbel et al., 2005), PI3K is required for *S. aureus* internalisation. In addition, because of the asymmetric localisation of TLRs on the apical and basolateral surfaces (Ioannidis et al., 2013), an invasion assay using polarised epithelial monolayers would closer reflect the physiological condition. This is further supported by the fact that *P. aeruginosa* binds to distinct receptors on the apical and basolateral surfaces, with different binding affinity (Bucior et al., 2010). Hence, a

future experiment is guaranteed to perform the invasion assay using epithelial cells grown on permeable membranes, and comparing invasion by infecting the monolayers from the apical and the basolateral surface.

As bacteria in biofilms are more resistant to antimicrobial substances and host clearance, biofilm formation is a key virulence factor for persistence and immune evasion. The strength of biofilm development by *S. aureus* is positively correlated with disease severity (Domenico et al., 2018). Both *S. aureus* and Bcc are known to form biofilm in close proximity to the apical mucosal surface (Krismer and Peschel, 2011; Schwab et al., 2002). Our results showed that *S. aureus* and Bcc developed differential amounts of biofilms. Some clinical isolates such as *B. multivorans* 13243, *B. cenocepacia* J2315, and MSSA NCTC 10788 formed more robust biofilms compared to non-invasive strains.

Heterogenicity was also observed in IL-8 production induced by *S. aureus* isolates. IL-8 is a biomarker for monitoring infections (Kraft et al., 2015). As the main receptor for recognising *S. aureus*, TLR2 activation depends on ligand abundance on the bacterial surface. Hence, differential activation of TLR2-mediated signalling by *S. aureus* clinical isolates may result from differences in bacterial metabolic activity, capsule expression and cell wall synthesis (expression of lipoproteins and SpA) (Hilmi et al., 2014; Peres et al., 2015). Bcc has prototypical ligands for TLR2, TLR4, and TLR5 (West et al., 2009). CF-related isolates *B. cenocepacia* J2315 and *B. multivorans* 13243 have been reported to induce high amounts of IL-8 in 16HBE cells, with a higher level induced by J2315 (Kaza et al., 2010). However, in our system, *B. cenocepacia* J2315 but not *B. multivorans* 13243 caused a significant increase in IL-8 production; thus, *B. cenocepacia* J2315 was chosen for further experiments.

The quantification of the IL-8 protein level was 24 h post-bacterial stimulation. However, the duration should be carefully decided, as protein levels are highly dynamic and regulated by mRNA level, translation, and degradation. Protein levels at steady state are dominated by mRNA levels, whereas under stress conditions, in addition to transcription changes, a delay occurs between changes in transcription and translation level, especially immune response proteins (Jovanovic et al., 2015; Liu et al., 2016). Hence, a time course of IL-8 protein level would be useful to decide the duration of bacterial stimulation. By doing so, the gap between different treatments might be larger.

IL-8 production is initiated by TLR-mediated downstream signalling cascades (Lim and Staudt, 2013). To elucidate which pathways are modulated by *S. aureus* and Bcc, phosphorylation of key signalling proteins following bacterial stimulation was investigated using western blot. In agreement with Gillette et al. (2013), *B. cenocepacia* J2315 led to

phosphorylation of p38, Erk and p65 NF-κB, whereas phosphorylation maintained the same when exposed to Staphylococcal strains and their supernatants. The minimal expression of TLR2 co-receptor CD36 restricts recognition of Gram-positive bacteria, which leads to airway epithelial cells hypo-responsive to Gram-positive bacteria but not Gram-negative bacteria (Mayer et al., 2007). On the other hand, p38 and Erk contribute to IL-8 mRNA stability in lung epithelial cells, supporting the observation that J2315 induces higher level of IL-8 compared to *S. aureus* isolates (Bhattacharyya et al., 2011).

6.2 Host response to polymicrobial infections and the comparison with monomicrobial infections

B. cenocepacia J2315 is strong IL-8 inducer on its own, and in a chronic *P. aeruginosa/ B. cenocepacia* co-infection model, significant higher amounts of cytokines and chemokines were induced by co-infection compared to single infections (Bragonzi et al., 2012). The net impact of polymicrobial infections leads to different host responses, and the consequences may be detrimental, insignificant or beneficial to the host (McArdle et al., 2018; Ramsey and Whiteley, 2009; Sibley et al., 2008). To elucidate the host response to *S. aureus/B. cenocepacia* co-infection, 2 isolates of *S. aureus* that downregulated IL-8 production without causing significant cell damage were chosen and supernatants from overnight cultures were prepared.

The data presented here is the first time to show the anti-inflammatory effect of *S. aureus* products when co-stimulated with live *B. cenocepacia* bacteria. Similar results were observed when Staphylococcal supernatants co-stimulated bronchial epithelial cells with supernatant of a close relative of *Burkholderia, P. aeruginosa* (Chekabab et al., 2015). Chekabab et al. (2015) demonstrated that the synthetic ligand for TLR1/2 induced IL-8 to the same level of as *P. aeruginosa* supernatant, and the response was abolished by *S. aureus* supernatant, whereas Beas-2B cells were hypo responsive to LPS or flagella. Further experiments confirmed that *S. aureus* supernatant blocked TLR2-mediated NF-κB signalling but not MAPK (Chekabab et al., 2015). To demonstrate which signalling pathway is involved in our system, we co-treated airway epithelial cells with *S. aureus* supernatant and *B. cenocepacia* J2315, followed by the assessment of phosphorylation using western blotting. Although not achieving statistical significance, *S. aureus* supernatant abolished phosphorylation of p38, Erk and p65 NF-κB. This is the first time showing the inhibitory effect of *S. aureus* on MAPK signalling.

The findings in this thesis demonstrate the antagonistic interaction between *S. aureus* and *B. cenocepacia* on inducing the pro-inflammatory response. By inhibiting MAPK and NF-

κB signalling cascades activated by *B. cenocepacia*, *S. aureus*-secreted products reduce IL-8 production in airway epithelial cells. This might shed light on a novel Staphylococcal immune evasion mechanism through inducing anti-inflammatory responses. Further, identification of the host target might provide a novel treatment for chronic inflammation resulted from excessive immune responses.

6.3 Interpretation of the host receptor for *S. aureus* anti-inflammatory factor(s)

TLRs are the major sensors for *B. cenocepacia,* and TLR2 is the most important PRR to detect *S. aureus* (Askarian et al., 2018). Chekabab et al. (2015) reported that *P. aeruginosa* activated TLR2 is blocked by *S. aureus* which subsequently subverts TLR2-mediated NF-κB signalling. To distinguish whether the effect was TLR-specific, upon stimulation with IL-1β and TNFα, airway epithelial cells were treated with *S. aureus* supernatants. In contrast, while IL-1β / TNFα induced the same level of IL-8 as *B. cenocepacia* J2315, *S. aureus* supernatants did not abolish IL-8 production. Hence, the anti-inflammatory effect of *S. aureus* is TLR-specific.

To illustrate which TLR is targeted by *S. aureus*, airway epithelial cells were exposed to *S. aureus* supernatants, together with TLR2/1, TLR4 and TLR5 agonists, respectively. However, although flagellin led to a significant increase in IL-8 production, none of the other TLR agonists elicited IL-8 to the same level as *B. cenocepacia* J2315 even at a high concentration. In addition, only flagellin significantly upregulated PI3K/Akt, MAPK and NF-κB signalling cascades in 16HBE cells. This is consistent with a previous study that only TLR5 but not TLR2 and TLR4 plays a central role in *B. cenocepacia*-induced pro-inflammatory responses (de C Ventura et al., 2008).

The hypo-sensitivity to TLR ligands have three possible explanations. The commercially purchased TLR ligands were isolated from other microorganisms. The acylation of LPS lipid A contributes to abnormal pro-inflammatory responses to whole-cell lysates and highly purified LPS from *B. cenocepacia* (De Soyza et al., 2004; Zughaier et al., 1999). Hence, stimulation with isolated TLR ligands from *B. cenocepacia* J2315 would closely reflect the host response to *B. cenocepacia.* Secondly, the hypo-responsiveness might result from restricted expression of TLRs. The epithelial cell line 16HBE exhibits a low but inducible level of TLR2 expression and a relatively higher level of TLR4 (Bailey et al., 2009; Pace et al., 2008), which explains hypo-responsiveness to Gram-positive bacteria compared to Gram-negative bacteria (Mayer et al., 2007). In addition, epithelial cells such as BEAS-2B are deficient in TLR2 co-receptor CD36, and TLR2/CD36 transfection results

in enhanced IL-8 production (Mayer et al., 2007). TLR5 is also present in 16HBE cells (Muir et al., 2004; Parker and Prince, 2013). Lastly, the differential responses to *B. cenocepacia* and TLR ligands might result from the additional receptors that are targeted by *B. cenocepacia* (Ganesan and Sajjan, 2011). For example, the O antigen of LPS from *B. cenocepacia* activates the inflammasome in a TLR4- and caspase-1-dependent manner (Kotrange et al., 2011). In addition, *B. cenocepacia* binds to TNFR1 and subsequently elicits robust IL-8 expression through an unknow ligand in CF airway epithelial cells (Sajjan et al., 2008). Hence, in addition to TLR activation, other host receptors might be involved in the disproportionate pro-inflammatory response to *B. cenocepacia.*

Further experiments were performed with TLR antagonists. TLR4 but not TLR5 inhibitor reduced IL-8 production elicited by *B. cenocepacia* to the same level of *S. aureus* supernatants. Multiple Staphylococcal products are known to antagonise TLRs, such as TLR2 by SSL3 (Bardoel et al., 2012). The anti-inflammatory molecules present in Staphylococcal supernatant might be PSMα, which consists of 20 to 30 amino acids with about 2 to 3 kDa peptides (Berube et al., 2014). PSMα1-α3 of *S. aureus* have shown to disrupt the interaction between TLR4 and high motility group box-1 protein, and subsequently inhibit NF-κB signalling in HEK-Blue hTLR4 cells (Chu et al., 2018). However, PSMα is able to activate TLR2 signalling (Armbruster et al., 2016) and HEK-Blue hTLR4 cells (InvivoGen, USA) are inactive to TLR2 ligands. What would be the net impact of PSMα if cells with the presence of TLR2 and TLR4 were used?

In our system, the downregulation on IL-8 production by blocking TLR4 was minimal but significant. Alternatively, following confirmation of TLR isoforms expression in airway epithelial cells, we could compare chemokine responses to *B. cenocepacia* using wild type cells and TLR isoform knockdown cells. By doing so, it would confer an unbiased actual response showing the net impact of Staphylococcal products. In addition, using live bacteria would reflect the actual ligands shed from intact bacteria compared to using TLR agonists.

As TLR4 is the only TLRs that requires both MyD88 and TRIF, this leads to a hypothesis that the anti-inflammatory effect of *S. aureus* results from blocking MyD88- and TRIF-dependent signalling. So far, YopJ from *Yersinia pseudotuberculosis* is the only bacterial effector that blocks type I IFN and cytokine responses through suppressing MyD88 and TRIF in DC and macrophages (Rosadini et al., 2015; Rosadini and Kagan, 2015). Hence, by knocking out MyD88 and TRIF, respectively, it could be possible to evaluate the role of MyD88 and TRIF when airway epithelial cells are co-stimulated with *B. cenocepacia* and *S. aureus* supernatants.

6.4 Characterisation of *S. aureus* immunomodulatory factor(s)

The nature of Staphylococcal products is defined as small (less than 3kDa), heat-stable, non-proteinaceous, and not cell wall-related. So far, the immunomodulatory molecules identified in *S. aureus* are mostly peptides or proteins (Bardoel et al., 2012; Chu et al., 2018; Tajima et al., 2006; Tajima et al., 2008; Chekabab et al., 2015), in contrast to our results. In addition to the possibility that the molecules are non-proteinaceous, they might be proteinase K-resistant peptides/proteins as protease-resistant proteins have been reported before, mainly in prion and *Mycoplasma* (Abd-Elhadi et al., 2015; Butler et al., 1991; Salta et al., 2014).

Alternatively, with the assistance of in silico prediction, liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) could be applied to identify the Staphylococcal secretomes that interact with human TLR4 (Boleij et al., 2011a; Fels et al., 2017). While computational technique provides rapid discovery of the potential candidates based on empirical data, in some cases the predicted secretomes might be poorly expressed. With major improvements in accuracy, efficiency and sensitivity, LC-MS/MS enables identification of small organic compounds such as proteins and sugars in the field of host-pathogen interactions (Portillo et al., 2011; Han et al., 2013; Schweppe et al., 2015). However, this technique is vastly restricted by the ratio of macromolecules between host and pathogen (for example, human proteomes have about 20457 proteins while the prokaryotic counterparts have about 4423 proteins), which results in a decrease in the number of identified pathogen proteins when analysing mixed host and pathogen lysates (Fels et al., 2017). Identification of Staphylococcal immunomodulatory molecules would illustrate a novel immune evasion mechanism contributing to *S. aureus* pathogenesis, and implicate novel anti-Staphylococcal therapeutic interventions.

6.5 Limitations

This study has several limitations. Firstly, one difficulty throughout the project was inadequate phosphorylation to bacterial stimuli. Phosphatase inhibitors were included in the lysis buffer to inhibit phosphatase activity during cell lysis. However, the inadequate response could not be ascribed to the endogenous immune tolerance since the positive control TNFα failed to cause a significant increase in phosphorylation in several blots. Other pharmacological reagents such as EGF and phorbol 12-myristate-13-acetate could be applied as positive controls for Akt and MAPK activation (Rice et al., 2002).

Alternatively, techniques to assess cumulative responses such as ELISA, intracellular flow cytometry or mass spectrometry, might provide a clearer readout.

Secondly, this project was limited to human airway cell lines. Although pulmonary epithelial cell lines and culture models are commonly used, there are inconsistencies between different phenotypes of cell lines (Kaza et al., 2010), and between cell lines and primary cells (Strobel et al., 2016) in the host response. Since polymicrobial respiratory infections are highly prevalent in CF individuals, CF-isolated primary tissues and immortalised cell lines would be critical for the understanding of biochemical, physiological and genetic mechanisms of host defence underlying CF defect (Gruenert et al., 2004).

Thirdly, this study requires confirmation using *in vivo* models. Using animal models would provide a panorama of the host response to *S. aureus*/Bcc co-infection. Moreover, there is limited research using rodent models to study polymicrobial infections. In this project, due to cell damage caused by *S. aureus*, pro-inflammatory responses to *S. aureus* and Bcc live bacteria were not evaluated. Alternatively, murine models could be applied to assess host responses to either acute or chronic polymicrobial infections, by intratracheal injection or agar bead-based method, respectively (Kukavica-Ibrulj et al., 2014). These models would be useful to study microbial clearance, inflammatory responses, mortality and lung histopathology.

Lastly, the interaction between *S. aureus* secreted products and TLR4 complex requires binding assays to be validated. Since antagonising TLR4, reduced *B. cenocepacia* J2315-induced pro-inflammatory response, it is plausible that Staphylococcal products competitively bind to TLR4 complex. However, this requires separating *S. aureus* supernatant using chromatography and binding assays to confirm TLR4 is the host target (Nicholas et al., 2017; Fuchs et al., 2018).

6.6 Final conclusions

This thesis demonstrates the disruptive effect of two microorganism species, *S. aureus* and Bcc on human airway epithelial monolayers, as well as their modulation on host signalling cascades. *S. aureus* and Bcc are also able to adhere to and enter into airway epithelial cells. *B. cenocepacia* J2315 exposure leads to phosphorylation of MAPK and NF-κB cascades, which results in substantial IL-8 production. However, this robust pro-inflammatory chemokine response is subverted by secreted molecules from *S. aureus,* partially through blocking TLR4 pathway. Preliminary characterisation describes the anti-inflammatory molecules as small, heat-stable, non-proteinaceous, and not cell wall-related factors. The findings might provide valuable insight into a novel Staphylococcal

immune evasion mechanism, as well as a resolution of tissue injury resulting from excessive or chronic inflammation.

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